IN VITRO PRODUCTION OF RABBIT MACROPHAGE TUMOR CYTOTOXIN: ITS ROLE IN MACROPHAGE MEDIATED TUMOR CELL KILLING

BY

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To my parents, Irving and Martha Fisch, for their years of love and concern and for setting the proper example.

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ABBREVIATIONS USED

BCG	Mycobacterium bovis, strain BCG
BLM	blood mononuclear cells
BLMCT	blood monocyte cytotoxin
BMC	bone marrow cells
BMCCT	bone marrow cell cytotoxin
BPTI	bovine pancreatic trypsin inhibitor
CT	cytotoxin(s)
FMEM	fortified Eagle's minimum essential medium
HEPES	N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid
LPS	lipopolysaccharide
MAF	macrophage activating factor
MEF	mouse embryo fibroblasts
MEM	Eagle's minimum essential medium
MPS	mononuclear phagocyte system
PEC	peritoneal exudate cells
PGE	prostaglandin E
PLC	pulmonary lavage cells
PLCCT	pulmonary lavage cell cytotoxin
PMSF	phenylmethyl sulfonylfluoride
RES	reticuloendothelial system
SBTI	soybean trypsin inhibitor
TNF	tumor necrosis factor
TNS	tumor necrosis serum
TNSCT	tumor necrosis serum cytotoxin
TPA	12-o-tetradecanoylphorbol 13-acetate

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Rabbit macrophages derived from pulmonary lavage cells (PLC), phorbol ester pretreated and washed bone marrow cells (BMC), or blood mononuclear cells (BLM), cultured in the presence of LPS, secreted tumor cell cytotoxins (CT) that were similar to each other and tumor necrosis serum cytotoxin (TNSCT).

All CT's had molecular weights of approximately 48,000 D by gel filtration and eluted from DEAE-Sephadex between 0.28 and 0.32 M salt. All were stable to 56°C for 60 minutes, but labile to 70°C for 20 minutes. Actinomycin D (AcD) enhanced sensitivity of L-929 cells to CT. $B16C_3$ melanoma cells and mouse embryo fibroblasts were resistant to CT.

By 3 hours in culture, all effector cells secreted detectable levels of CT. Titers increased for PLC and BLM between 3 and 5 hours. After that time, BLM titers remained constant through 24 hours. PLC titers rose again from 18 to 30 hours. BMC cultures

demonstrated increasing titers from 3 through 14 hours. After that, no increase in titer was demonstrated. No additional CT production could be demonstrated after 30 hours in BMC or PLC cultures, despite a change to fresh medium with LPS.

On an adjusted basis of 1×10^6 macrophages/ml, PLC produced 4 to 25 times more CT than BMC or BLM after 28 hours culture.

CT production by LPS-activated PLC could occur in serum-free medium. Fetal bovine serum (10%) enhanced production up to 4-fold. Bovine serum (>4%), or prostaglandin E_2 (10^{-6}M) inhibited CT production. AcD (1 µg/ml) added with LPS inhibited CT production (>95%) by PLC. Delaying addition of AcD after LPS demonstrated messenger RNA production for CT was completed by 2 to 6 hours.

AcD pretreatment of L-929 cells (2 µg/ml, 2 to 3 hours) followed by washing enhanced their sensitivity to PLC killing. From this, a plaque assay was generated demonstrating a linear relationship between number of PLC plated and plaques formed. Therefore, 1 PLC was capable of forming a plaque. Time lapse cinematography demonstrated that contact between PLC and targets was unnecessary for target cell killing.

$B16C_3$ cells and L-929 cells selected for resistance to TNSCT were resistant to PLC mediated cytotoxicity.

The conclusion drawn is that secreted CT may play a major role in macrophage mediated tumor cytotoxicity.

INTRODUCTION

The problems encountered in biological disciplines are often frustrating and at times seemingly insurmountable. This is the case in the broad field of tumor immunology, an area filled with complexity and apparent contradiction.

Immune System Components: In Vitro Cytotoxicity for Tumors

The interactions of the various humoral and cellular components of the immune system with tumors as well as with each other *in vivo* and *in vitro* have been well documented. This has been described as a "network" (Jerne, 1974, 1977) as well as a surveillance system seeking out tumors as they arise within the host (Thomas, 1959; Burnet, 1967, 1970). Some investigators seriously question the validity of these sweeping concepts in the face of spontaneously arising tumors (Hewitt et al., 1976). Evidence also exists illustrating that some tumors grow as a result of an immune response (Prehn, 1976).

Despite these difficulties, there has been much effort put forth to investigate and understand just what the components of the immune system can do when confronted with tumors both in the test tube and in the tumor bearing host. More specifically, sensitized cytotoxic T lymphocytes (Brunner and Cerottini, 1971), natural killer lymphocytes (Herberman et al., 1975), and armed and activated macrophages (Alexander, 1974) represent cell types that can directly kill tumor cells subsequent to contact with them. Some of the reported humoral activities cytotoxic for

tumor cells include specific antibody and complement (Hellstrom et al., 1968), lymphotoxin, a product of lymphocytes (Granger and Kolb, 1968), tumor necrosis factor, and cytotoxic factor (Carswell et al. 1975), presumed macrophage products (Matthews, 1978; Mannel et al. 1979, 1980a, 1980b). Examples of humoral and cellular elements working together to destroy tumor cells include specific antibody with nonimmune lymphocytes (Pollack et al., 1972) and specific antibody with macrophages (Whitcomb, 1979).

The Mononuclear Phagocyte System: A Macrophage Network

The mononuclear phagocytes or macrophages represent a large, morphologically heterogeneous group of cells found widely distributed throughout the body (Vernon-Roberts, 1972). Metchnikoff (1905) recognized the presence of mononuclear cells in the blood, lymph, as well as throughout the various organs, that could engulf small particles such as bacteria by projecting ameboid processes. He coined the term "phagocytosis" (from the Greek word, "phagein," to eat) and designated the large free and fixed phagocytic cells as "macrophages." He also demonstrated the significance of these phagocytic cells concerning host defense by reconfirming their presence in inflamed tissue as well as noting their phagocytic capacity for bacteria.

Utilizing vital dyes which are preferentially taken up by macrophages and to a lesser extent by other types of cells, Aschoff (1924) demonstrated the distribution throughout the body of a functional system of cells which included macrophages. He referred to this overall

network of cells as the "reticuloendothelial system (RES)." It was believed that these cells were responsible for the formation of the extrasinusoidal solid pulp of the lymph nodes and spleen (reticulum). In addition, they were found lining the sinusoids of lymph nodes and other organs (endothelium). Additional investigations extended the functional RES to include the microglia of the central nervous system (del Rio Hortega and de Asua, 1924) and blood monocytes (Marshall, 1956).

Later studies reviewed by van Furth et al. (1975) revealed that the original concept of the RES as a unified system is inappropriate in that it really consists of at least two distinct systems. One of these, the "RES proper" is composed of cells that are nonphagocytic and make up the framework of connective tissue. Distinct from these cells are the macrophages, which often exist either fixed or free, in close proximity to connective tissue. As a result, the term "mononuclear phagocyte system" (MPS) has been proposed to denote a functional network made up exclusively of macrophages (van Furth et al., 1975).

As previously mentioned, macrophages display considerable morphological heterogeneity (Vernon-Roberts, 1972). This no doubt created considerable confusion among early investigators regarding what was and was not to be considered a macrophage. Perhaps the most enlightening studies concerning this problem, as well as revealing the source of macrophages, have dealt with their ontogeny. Macrophages originate from a common source in the bone marrow, enter the circulation and finally pass into the tissues, changing their morphological and many of their functional characteristics as they proceed (van Furth et al.,

1975; van Oud Alblas and van Furth, 1979). Therefore, the varied descendants of the bone marrow precursor merely represent different stages or various end cells of a progressive differentiation process.

Aside from their mononuclear appearance, intense phagocytic capability and common origin, macrophages share an array of other morphological, chemical and functional characteristics. Included among these properties are the ability to adhere tightly to glass, motility, possession of abundant lysosomes containing various enzymes, production of various immune modulators, complement components, superoxide free radical and hydrogen peroxide and possession of Fc and complement receptors (Eisen, 1980). In additional to morphological features, heterogeneity among macrophages can be extended to these properties as well since different macrophages vary qualitatively and quantitatively with respect to these characteristics (van Oud Alblas and van Furth, 1979; Keller, 1980). This overall heterogeneity reflects the existence of clearly separable subpopulations (Keller, 1980) with either distinct genetic differences or changes that come about with pluripotent macrophage maturation or activation.

Many of these properties contribute to the ability of macrophages to either act alone or in conjunction with other cells or products of the "immune system" (T and B lymphocytes, antibody and complement) to help maintain a state of homeostasis within the host. More specifically, this involves elimination of exogenously introduced toxins, invading microorganisms, or other foreign antigens, as well as the destruction and eventual elimination of host cells that are effete or have undergone certain alterations. This latter case includes the changes seen in cells associated with neoplasia.

Significance of the Mononuclear Phagocyte System in Tumor Regression

Nearly a century ago, Coley (1891) reported on the palliative (and occasional curing) effects of using bacterial culture filtrates to treat patients with neoplastic disease. The rationale for this treatment of neoplasia was based on the observation (made long before that) that cancer patients with concurrent infectious disease often displayed regressing neoplasia or tumors that advanced less rapidly than those of cancer patients without concurrent infection. More recent reviews (Pearl, 1929; Jacobsen, 1934; Nauts et al., 1953) have discussed related phenomena in detail.

Later investigations revealed that the MPS could expand and increase its activity during the course of a neoplastic disease (Old et al., 1960) or following the administration of such agents as Mycobacterium bovis, strain BCG (Old et al., 1960; Leake and Myrvik, 1968), zymosan (Old et al., 1960), or Corynebacterium parvum (Halpern et al., 1974). Similar studies also showed that such agents could protect challenged animals against transplanted tumors or increase the survival time of these animals (Old et al., 1959; 1960).

Further evidence that contributed credibility to the macrophage as a critical component of the in vivo effector response to tumors was later provided. Macrophages could be made nonspecifically cytotoxic to tumor cells by culturing them in the presence of endotoxin (Alexander and Evans, 1971), or supernatant fluids from antigen sensitized lymphocyte cultures (Piessèns et al., 1975; Leonard et al., 1978). Alternatively, macrophages could be rendered specifically cytotoxic or "armed" to a

particular tumor by cocultivating them with spleen lymphocytes from an animal previously inoculated with the tumor (Alexander, 1974). These macrophages could then be rendered nonspecifically cytotoxic (activated) to different tumors by continued cocultivation with the original immunizing tumors (Alexander, 1974).

More convincing evidence indicating that macrophages play a significant role in the overall scheme of tumor defense mechanisms was revealed by their actual presence within tumors (Evans, 1972; Alexander, 1974; Eccles and Alexander, 1974). Furthermore, tumor regression could be correlated with macrophage infiltration (Russell et al., 1976).

The Process of Macrophage Activation

The term "activated" as it pertains to the macrophage was introduced by MacKaness (1970) and it is a term which has received wide usage by many investigators who study the macrophage. Depending upon one's interests, the activated macrophage possesses many characteristics which distinguish it from a macrophage which is not activated. Allison and Davies (1975) have described biochemical and biological changes in macrophages undergoing activation. These include increases in the following: glucose oxidation, lysosomal enzyme levels, synthesis of membrane components, pinocytosis, phagocytosis, adhesion, spreading and motility. In addition, activated macrophages, as opposed to resting macrophages, can inhibit the proliferation of and have demonstrated cytotoxicity for tumor cells (Allison and Davies, 1975; Hibbs, 1976; Keller, 1980).

The cytotoxicity that activated macrophages demonstrate for transformed cells is most interesting. Aside from being nonspecific

as seen by the ability of activated macrophages to kill different types of tumors from allogeneic, syngeneic or xenogeneic sources (Keller, 1980), most nontransformed cells are not killed by activated macrophages (Hibbs, 1974a; Keller, 1980). Therefore, the activated macrophage appears to be capable of distinguishing some common feature(s) of the tumor state from that of normal cells. Currently, the exact distinction between tumor and nontransformed cells made by the activated macrophage is unknown. However, some evidence suggests the difference lies within the plasma membrane (Hibbs, 1974a).

The process of macrophage activation is not a simple, absolute, one-step event, but involves a cascade of transient, intermediary reactions (Hibbs et al., 1980; Meltzer et al., 1980) dependent upon the presence of signals in the local environment of the macrophage (Hibbs et al., 1977). Some of these signals are stimulatory while others are inhibitory to macrophage activation (Hibbs et al., 1977; Chapman and Hibbs, 1977). Ruco and Meltzer (1978) have extended this model by describing it as a series of "priming" signals followed by activation signal(s).

As mentioned previously, some of the signals that can affect the state of the macrophage and modulate activation have been identified. They include endotoxin (Alexander and Evans, 1971), macrophage activating factor, a T lymphocyte product (Leonard et al., 1978) and type I interferon (Schultz et al., 1977; Jett et al., 1980). As work in this field progresses, it is becoming more and more evident that many of these activation signals are related to one another as part of the macrophage activation cascade, particularly as priming or activation signals.

This is the case concerning macrophage activating factor (MAF) and the active component of endotoxin, lipopolysaccharide (LPS). It has been established using highly purified materials that MAF alone cannot activate macrophages, but merely sensitizes or "primes" them to the activating capabilities of LPS (Weinberg and Hibbs, 1979; Weinberg and Hibbs, 1980; Hibbs et al., 1980; Weinberg, 1981; Pace and Russell, 1981). Earlier studies concerning these two signals in this scheme were complicated by the presence of small amounts of LPS contaminating the lymphokine preparations as well as other materials (Hibbs et al., 1980). Aside from clarifying the roles of MAF and LPS in the macrophage activation scheme, many of these studies demonstrate the importance of determining the presence of contaminating LPS when working with macrophages (Levin et al., 1970; Fumarola, 1981).

Despite the apparent clarity of this simple model utilizing MAF (a priming signal) and LPS (a triggering signal), there are many points regarding macrophage activation and its associated signals that are still not clear. First, it is apparent that LPS is not the only triggering signal. This has been demonstrated in several studies which either exclude LPS from cultures or utilize macrophages derived from mice that respond very poorly to LPS (Hibbs, 1976; Schultz and Chirigos, 1980; Boraschi and Tagliabue, 1981). Secondly, type I interferon has been described as a priming signal distinct from MAF (Boraschi and Tagliabue, 1981) as well as serving as the endogenous macrophage generated mediator for LPS induced triggering of macrophage activation (Schultz and Chirigos, 1979). Further studies are needed to determine the identity of other

triggering signals as well as the precise role of interferon in the scheme of macrophage activation.

The Elicitation of Serum Tumor Necrotic and Cytotoxic Activities

Many of the foregoing mechanisms concerning macrophage activation are now believed to represent accurate *in vitro* correlates of host mediated responses to such agents as BCG, zymosan, *Corynebacterium parvum* and LPS. Although the early investigators were not aware of the details behind the host's response to these agents, it was quite apparent that they had value in inducing favorable clinical responses in tumor bearing animals. Ribi et al. (1975) gave tumor bearing guinea pigs both BCG and LPS. The results of this double regimen were rather impressive. Test animals that had received both BCG and LPS displayed a survival rate of about 90% compared to a survival rate of about 66% for those receiving BCG alone.

Carswell et al. (1975) found that they could cause a dramatic necrosis of transplanted subcutaneous tumors in otherwise unmanipulated mice that were injected with sera from other mice, rabbits, or rats that had previously been inoculated intravenously with BCG, *Corynebacterium* species or zymosan followed by an intravenous injection of LPS 2 weeks later. They called the agent(s) in the sera responsible for this phenomenon, tumor necrosis factor (TNF). It was also demonstrated by the same group (Carswell et al., 1975; Helson et al., 1975; Green et al., 1976) that crude tumor necrosis serum (TNS) or material that was partially purified from TNS could kill many but not all types of tumor cells *in vivo* and *in vitro* while being nontoxic for mouse embryo

fibroblasts *in vitro*. Others have reported similar findings for TNS derived from the mouse (Mannel et al., 1980a; Mannel et al., 1980c) and rabbit (Matthews and Watkins, 1978; Ostrove and Gifford, 1979). In addition, some of these as well as other studies demonstrate that neither tumor necrosis or *in vitro* cytotoxicity are due to LPS (Carswell et al., 1975; Oettgen et al., 1980). LPS levels in the serum transferred from donor animals are substantially below the levels required to cause tumor necrosis. Furthermore, LPS is not toxic for tumor cells *in vitro* and has a profile distinctly different from that of TNF regarding other biological activities (Oettgen et al., 1980).

Biochemical and Physical Properties of Tumor Necrosis Factor and Cytotoxins in Tumor Necrosis Serum

Green et al. (1976) partially purified TNF from mouse TNS and determined it to have a molecular weight of 125,000 to 150,000 D by gel filtration. Further studies in the mouse (Mannel et al., 1980a; Mannel et al., 1980c; Oettgen et al., 1980) demonstrated the molecular weight of the cytotoxic activity (CT) and TNF respectively (by gel filtration) to be about 60,000 D, after exposure to high salt concentrations (0.2 M or greater). This may indicate ionic aggregation of polymeric subunits or aggregation with some other molecular species found in the serum. Other properties of mouse TNF and tumor necrosis serum cytotoxin (TNSCT) include stability to 56° C exposure for 30 minutes, an isoelectric point of pH 4.8, pronase sensitivity, and the presence of sialic acid and galactosamine residues (Green et al., 1976; Mannel et al., 1980c; Oettgen et al., 1980).

Studies in the rabbit employing gel filtration have determined the molecular weight of TNSCT to be 55,000 D (Ruff and Gifford, 1980) and 39,000 D (Matthews et al., 1980). The two groups reported approximately the same molecular weight (67,000-68,000 D) utilizing polyacrylamide gel electrophoretic techniques. The consistently higher molecular weight as determined by electrophoresis over that of gel filtration could possibly be explained by alteration of configuration of the cytotoxic molecule by denaturing conditions (Ruff and Gifford, 1980) or interaction of the molecule with the electrophoretic matrix resulting in retardation of migration (Matthews et al., 1980). The reasons for the difference between the two groups concerning the molecular weight as determined by gel filtration are unclear. Additional characteristics of rabbit TNSCT include relative stability to 70°C for 20 minutes (Matthews et al., 1980), an isoelectric point of pH 5.1, and high sensitivity to pronase and relative insensitivity to trypsin (Ruff and Gifford, 1980; Matthews et al., 1980). These and other studies (Ruff and Gifford, 1981) suggest that the rabbit CT molecule does not contain large amounts of exposed carbohydrate contains little or no carbohydrate, as based upon inability to bind cytotoxic activity to various plant lectins.

Are Tumor Necrosis Serum-Derived Necrotic and Cytotoxic Activities Mediated by the Same Effectors?

It is tempting to speculate from results of many of the aforementioned studies that the TNS agents responsible for tumor necrosis (TNF) and *in vitro* cytotoxicity are one and the same. However, the complexity of TNS is underscored by the demonstration that a variety of biological products such as interferon (Sauter and Gifford, 1966), B cell differentiating factor (Hoffman et al., 1976), bone marrow colony

stimulating factor (Hoffman et al., 1977) and lysosomal enzymes (Sauter and Gifford, 1966; Green et al., 1976; Old, 1976) are released into the serum under conditions used for induction of tumor necrosis. It is therefore possible that many mediators may contribute to tumor necrosis and cytotoxicity and the mediator(s) responsible for one of these phenomena may not be responsible for the other.

Initial studies employing crude TNS (Carswell et al., 1975) implied that a single entity, TNF, could be responsible for both in vivo tumor necrosis and in vitro cytotoxicity. As a result, many of the studies that followed exclusively utilized either the in vivo assay for necrosis Green et al., 1976) or an in vitro assay for cytotoxicity (Matthews et al., 1980; Mannel et al., 1980d) to study purified effector material. It was felt that either assay probably reflected the activity of the same effector. Along these lines, Oettgen (1980) and Kull and Cuatrecasas (1981), working in the mouse system, were able to partially copurify both cytotoxic and necrotic activities from TNS. Additional studies demonstrated that partially purified CT derived from rabbit TNS could elicit tumor necrosis (Ruff and Gifford, 1980). The data of Kull and Cuatrecasas (1981) are particularly interesting because they demonstrated material with cytotoxic activities having molecular weights of 50,000, 160,000 and 225,000 D, but only the 160,000 D containing fractions also displayed necrotic activity. In addition, they found that high molecular weight species persisted after salt fractionation, and gel filtration of the high molecular weight form in high salt led to loss of activity without an increase in activity of low molecular weight species (50,000 D). This

is contrary to the hypothesis concerning high salt deaggregation of high molecular weight forms (Mannel et al., 1980a; 1980c).

The results of the aforementioned studies do not definitively demonstrate whether or not TNS necrotic and cytotoxic activities can be attributed to a single effector molecule. Further investigation is needed to clarify this point.

Tumor Necrosis and Tumor Regression

Early studies utilizing animals with transplanted subcutaneous tumors demonstrated a "hemorrhagic necrosis" of their tumors after being administered bacterial culture filtrates of such organisms as Escherichia coli (Gratia and Linz, 1931). Later, Shear and Turner (1943) purified a polysaccharide containing lipid material from Serratia marcescens (now known to be LPS) which alone could cause a rapid and dramatic hemorrhagic necrosis of tumors. Although the mechanism behind this phenomenon remains unclear at this time, there is evidence suggesting an LPS induced vasoconstriction and intravascular coagulation within the tumor (Algire et al., 1952) as well as a need for lymphoreticular cells that are sensitive to LPS (Mannel et al., 1979). As already mentioned, a similar phenomenon can be seen in tumor bearing animals administered TNS (Carswell et al.,1975) or partially purified TNF (Ruff and Gifford, 1980). It is not yet clear (but is circumstantially possible) that TNF plays a role in the tumor necrosis induced by LPS alone. As rapid (often less than a day) and dramatic as this phenomenon is, a ring of viable tumor cells usually survives at the periphery of the neoplasm to grow and kill the host (Shear, 1943). This demonstrates that tumor necrosis

in itself is usually insufficient to lead to total recovery from neoplastic disease.

Tumor regression, on the other hand, involves all those events which contribute and ultimately lead to total recovery of the host from naturally occurring or experimentally induced tumors. It is possible that tumor necrosis may contribute to or allow subsequent tumor regression to take place more readily by destroying those parts of the tumor (the center) which effector mechanisms (such as activated macrophages) may have difficulty reaching.

As previously discussed, much evidence has been provided concerning the significance of the macrophage in overall tumor regression (Evans, 1972; Alexander, 1974; Eccles and Alexander, 1974; Russell et al., 1976). It is possible that in many cases, the macrophage may be directly involved in tumor regression that has been attributed to other types of effector cells (natural killer cells, cytotoxic T lymphocytes). As an example, studies by Berendt et al., (1978a; 1978b) have indicated that LPS induced tumor regression requires concomitant T cell immunity and that only those tumors that are immunogenic enough to evoke T cell immunity will regress. At first inspection it is difficult to envision a role for macrophages in such a scheme. However, the macrophage activation scheme involves a series of complex events dependent upon various signals such as MAF, a T lymphocyte product (Leonard et al., 1978; Weinberg and Hibbs, 1979). It is quite possible that macrophages may be "primed" by MAF produced by immunized T cells and subsequently activated by the administered LPS, thereby allowing them to be responsible at least in part for LPS induced tumor regression.

Activated Macrophages Produce Soluble Cytotoxic Activity in Vitro

The involvement of the macrophage in the production of tumor necrotic and cytotoxic serum activities is suggested by the coincident hyperplasia of the MPS accompanying the administration of many TNS eliciting agents (Carswell et al., 1975; Old, 1976). In addition, evidence demonstrating that macrophages can kill tumor cells *in vitro* (Alexander, 1974; Hibbs, 1976) prompted studies that sought to better understand the relationship of TNF and TNSCT to activated macrophages.

Studies in the mouse have demonstrated that LPS sensitive cells are needed to induce TNF in serum (Mannel et al., 1980d) and that biochemical and physical properties of serum-derived TNSCT are similar to those of CT derived from cultures of activated macrophages and macrophage cell lines (Mannel et al., 1980a, 1980b, 1980d). In addition, these same studies demonstrated that a rabbit antiserum directed against serum-derived TNSCT inhibited all soluble cytotoxicity generated in the macrophage cultures.

Working in the rabbit, Matthews (1978) has demonstrated that blood monocytes produce soluble tumor CT *in vitro*. This CT has striking similarity to rabbit TNSCT in terms of selective toxicity for various tumor cells and biochemical characteristics.

Purpose of These Studies

The aims of these studies are as follows:

1. To study the production of rabbit macrophage CT *in vitro* utilizing macrophages from different sources (pulmonary alveolar, bone marrow, and blood mononuclear cells).

2. To compare physicochemical and biological properties of these CT with one another as well as with those of TNSCT.

3. To determine the significance these CT play in macrophage mediated tumor cytotoxicity.

MATERIALS AND METHODS

Animals

New Zealand white female rabbits, ranging in age from 3 to 5 months (mean weights of 3 to 5 kilograms) were employed. Mice (C_3H/HeN and $C_{57}B1/6$, female) used ranged in age from 6 to 12 weeks. All animals were obtained from commercial sources and housed and fed conventionally.

Cell Lines

L-929 cells (a C_3H mouse cell line) were grown in Eagle's minimum essential medium (MEM) supplemented with 10% bovine serum and 250 units of penicillin/ml and 125 µg of streptomycin/ml. $B16C_3$ melanoma cells (a $C_{57}B1/6$ mouse cell line) were grown in fortified MEM supplemented with 10% fetal bovine serum. Fortified MEM contained 2x concentrations of essential and nonessential amino acids and vitamins. Glutamine and sodium pyruvate were also added. Primary mouse embryo fibroblasts (MEF) were prepared by mincing near term embryos followed by trypsinization for 60 minutes (0.1% trypsin, 0.04% ethylene diamine tetraacetic acid), washing in Gey's balanced salt solution and culturing in glass bottles containing FMEM with 10% fetal bovine serum.

Culture Systems

Media

These have been described above (see Cell Lines). For use in cultures where macrophages were employed, N-2-hydroxyethyl piperazine-N'-2-ethane sulfonic acid (HEPES buffer, Calbiochem, LaJolla, CA) was added at .03M.

Sera

Two lots of bovine sera were used: Gibco (Grand Island, NY), lot number R290222 and Sterile Systems, Inc. (Logan, UT), lot number 400168. The latter was certified to contain less than 1 ng lipopolysaccharide/ml. Unless otherwise stated, this was the lot used. One lot of fetal bovine serum was used for all experiments in this study (Kansas City Biological, Lenexa. KS).

Lipopolysaccharide-Free Culture System

Using a Limulus amebocyte lysate reagent kit (Pyrogent, Mallinckrodt Inc., St. Louis, MO) to test various media components for contaminating lipopolysaccharide (LPS), a culture medium was established which contained less than 0.025 ng LPS/ml (see Table III for the various materials screened). This "LPS-free" culture system was used for most of the studies to be discussed.

Effector Cells (Macrophages)

Rabbit Macrophage Sources

Rabbit macrophages were derived from three sources, pulmonary lavage cells (PLC), bone marrow cells (BMC), and blood mononuclear cells (BLM). For harvesting PLC and BMC, rabbits were anesthetized with a combination general anesthetic consisting of ketamine hydrochloride (Bristol Laboratories, Syracuse, NY) and xylazine hydrochloride (Cutter Laboratories, Shawnee, KS) followed by intravenous air embolism sacrifice. After hair removal and cleansing of the skin with 70% ethanol, the trachea and/or femurs were exposed. For PLC harvest, the trachea was transected and the lungs flushed out with six aliquots of 50 ml cold sterile pyrogen-free saline using a sterile disposable syringe. Cells were immediately removed from the cold saline by centrifugation and resuspended

in cold MEM supplemented with 10% fetal bovine serum and .03M HEPES buffer. Cells were generally washed twice by centrifugation in medium. About 20 to 40 x 10^6 PLC were obtained per rabbit. For BMC, femurs were removed and trimmed free of as much soft tissue as possible. After the bones had been rinsed well with sterile, pyrogen-free saline, the ends were removed with sterilized bone cutters and the contents of the marrow cavity flushed out with FMEM supplemented with 10% fetal bovine serum and .03M HEPES buffer, using a 16 gauge needle attached to a 20 ml sterile disposable syringe. Up to 2 x 10^9 bone marrow cells were obtained per rabbit.

For harvesting BLM, rabbits were anesthetized and bled from the marginal ear vein into sterile tubes containing pyrogen-free heparin. After collection, the blood was underlayered with half its volume of Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) and centrifuged at 400 g for 40 minutes at room temperature. The leukocytes at the interface were collected, washed 3 times with cold pyrogen-free saline and suspended in MEM, supplemented as for PLC. Generally, 50 to 100 x 10^6 BLM were obtained from one rabbit. Where necessary to minimize effector cell clumping and to insure a majority of single effector cells (see Plaque Assay), PLC were pushed through a 19, 23 and finally a 25 gauge hypodermic needle, using a syringe.

Leake and Myrvik (1968) have described a technique to significantly increase the yield of PLC from rabbit lungs. When this technique was modified by intravenous injection of 0.25 ml Freund's complete adjuvant (Gibco, Grand Island, NY) instead of Mycobacterium bovis, strain BCG (Leake and Myrvik, 1968), PLC yields were significantly increased up to about 200 x 10^6 per rabbit, if cells were harvested 2 to 3 weeks after the injection. These rabbits will be referred to as "primed" throughout

these studies. Those rabbits which were previously unmanipulated prior to sacrifice will be referred to as "normal."

Mouse Macrophage Source

Mouse macrophages were derived from peritoneal exudate cells (PEC). C_3H/HeN and $C_{57}B1/6$ mice were injected intraperitoneally with 2.5 to 3.0 ml of fluid thioglycollate medium (Bacto, Difco, Detroit, MI). Three days later, the mice were sacrificed by cervical dislocation and peritoneal exudate cells (PEC) harvested by washing out the peritoneal cavities with 8 to 10 ml of cold medium. The cells were washed twice by centrifugation and resuspended in fresh medium. As with rabbit PLC, where necessary to minimize cell clumping and insure a majority of single cells, PEC were pushed through 19, 23 and finally 25 gauge hypodermic needles, using a syringe.

Activation of Macrophages in Culture

LPS (Escherichia coli 055:B5, Westphal, Difco, Detroit, MI) was used to induce macrophage activation in all cultures. Unless otherwise stated, culture conditions were as follows: for mouse PEC or rabbit PLC and BLM, LPS was added at 10 µg/ml in MEM, supplemented with 10% fetal bovine serum plus .03M HEPES buffer; for rabbit BMC, cells were cultured in FMEM supplemented with 10% fetal bovine serum plus .03M HEPES buffer. Furthermore, concerning BMC, the phorbol ester, 12-o-tetradecanoylphorbol 13-acetate (TPA) was added at 0.1 or 1 µg/ml and the cells generally cultured overnight, after which time they were washed free of TPA (4 to 6 times) and refed with fresh medium containing LPS (see Increasing the Macrophage Content of Bone Marrow Cultures, under Results). Unless otherwise stated, all effector cells were cultured at 1×10^6 cells/ml.

Viability of all effector cell populations was determined by trypan blue dye exclusion. All effector cells used had 95% viability or greater by this criterion and were always cultured at a density based on the percentage of viable cells.

Identification of Macrophages in Effector Cell Populations

Identification of macrophages was based on morphological criteria (Giemsa's blood stain), phagocytic capability of latex beads (Dow Chemical Co., Indianapolis, IN) and peroxidase content (Leukocyte Peroxidase, Histozyme Kit No. 390A, Sigma Chemical Co., St. Louis, MO).

Production of Tumor Necrosis Serum

The basic technique has been described by Carswell et al. (1975). Rabbits were injected intravenously with viable BCG (Tice strain, 3 x 10^8 organisms). Two to three weeks later, LPS was injected intravenously and the rabbits bled 2 to 3 hours later. The blood was allowed to clot and the serum was withdrawn, further clarified by centrifugation, and aliquoted. Aliquots were frozen at -20°C until used. Once thawed, tumor necrosis serum (TNS) could maintain cytotoxicity titer (see assays) when kept at 4°C for several months.

Production of Rabbit Macrophage Cytotoxins

Rabbit PLC, BLM or TPA-pretreated and washed BMC were cultured in appropriate medium containing LPS. (Occasionally BMC were cultured in 175 cm^2 plastic tissue culture flasks at 2 to 5 x 10^6 cells/ml to increase titer yields. Otherwise, PLC and BLM were generally cultured at 1 x 10^6 cells/ml in 25 cm^2 plastic tissue culture flasks or 24 well plastic tissue culture plates.) After an appropriate time at 37°C, supernatants were harvested and cells removed by centrifugation.

Supernatants were titered and used within 48 hours if stored at 4°C (no appreciable loss of titer occurred if stored at this temperature for this time) or frozen at -80°C. Supernatants frozen at -80°C maintained titers for several months. Cytotoxins (CT) generated by rabbit macrophages are designated as follows: pulmonary lavage cells (PLCCT), TPA-pretreated and washed bone marrow cells (BMCCT), and blood mononuclear cells (BLMCT).

Cytotoxicity Assays

Photometric Assay

A basic photometric technique has been previously described (Ruff and Gifford, 1980). L-929 cells were plated in 96-well tissue culture trays at 50,000 cells/0.1 ml to establish a dense monolayer. Dilutions of CT in 0.1 ml were added along with actinomycin D (Calbiochem, LaJolla, CA) to give a final concentration of 1 µg/ml and the trays incubated at 37°C for 18 hours in a humidified 5% CO_2 atmosphere. After the culture period, the plates were stained for 10 minutes with crystal violet (0.2% in 2% ethanol), washed with water and allowed to dry.

Destruction of monolayers was evaluated by placing trays on an X-ray viewing screen and measuring light transmission through monolayers with a photocell. The reciprocal of the dilution of CT required to kill 50% of the targets was taken as the 50% endpoint (S_{50}). Two kinds of photocells were used in these studies. A manually operated photocell unit was constructed using a photoconductive cell (cadmium sulfide, No. 05HL, Clairex Electronics, Mt. Vernon, NY). The cell was cemented to a small piece of copper tubing that had an outside diameter approximating the inside diameter of culture wells. The photocell was attached to a multipurpose meter and read as a variable resistance at a fixed voltage as a function of light transmitted through the monolayers. For convenience

an automated photocell (Titertek Multiskan, Flow Laboratories, Inc., Inglewood, CA) was also used for some of the experiments. Figure 1 compares the two photocells reading the same sample wells. They are fairly similar, although it appears that the automated photocell is slightly more sensitive and can detect very small numbers of target cells retained at lower CT dilutions that the manual photocell cannot. Nevertheless, since an entire experiment was always read with the same photocell, and the enhanced sensitivity demonstrated by the automated photocell was not much greater than the manual one (about 15% greater, see Figure 1), this difference in photocell sensitivity is insignificant.

This photometric technique was slightly modified to measure effector cell killing of targets. The only difference from the aforementioned procedure used to titer CT involved adding actinomycin D at 2 µg/ml for 2 to 3 hours, followed by removal of this drug from the medium by decanting, washing the monolayer with medium and replacing with fresh medium containing effector cells. It was previously determined that 2 washings were sufficient to remove virtually all extracellular actinomycin D (washings at this point could not inhibit H^3-uridine incorporation into L-929 cells).

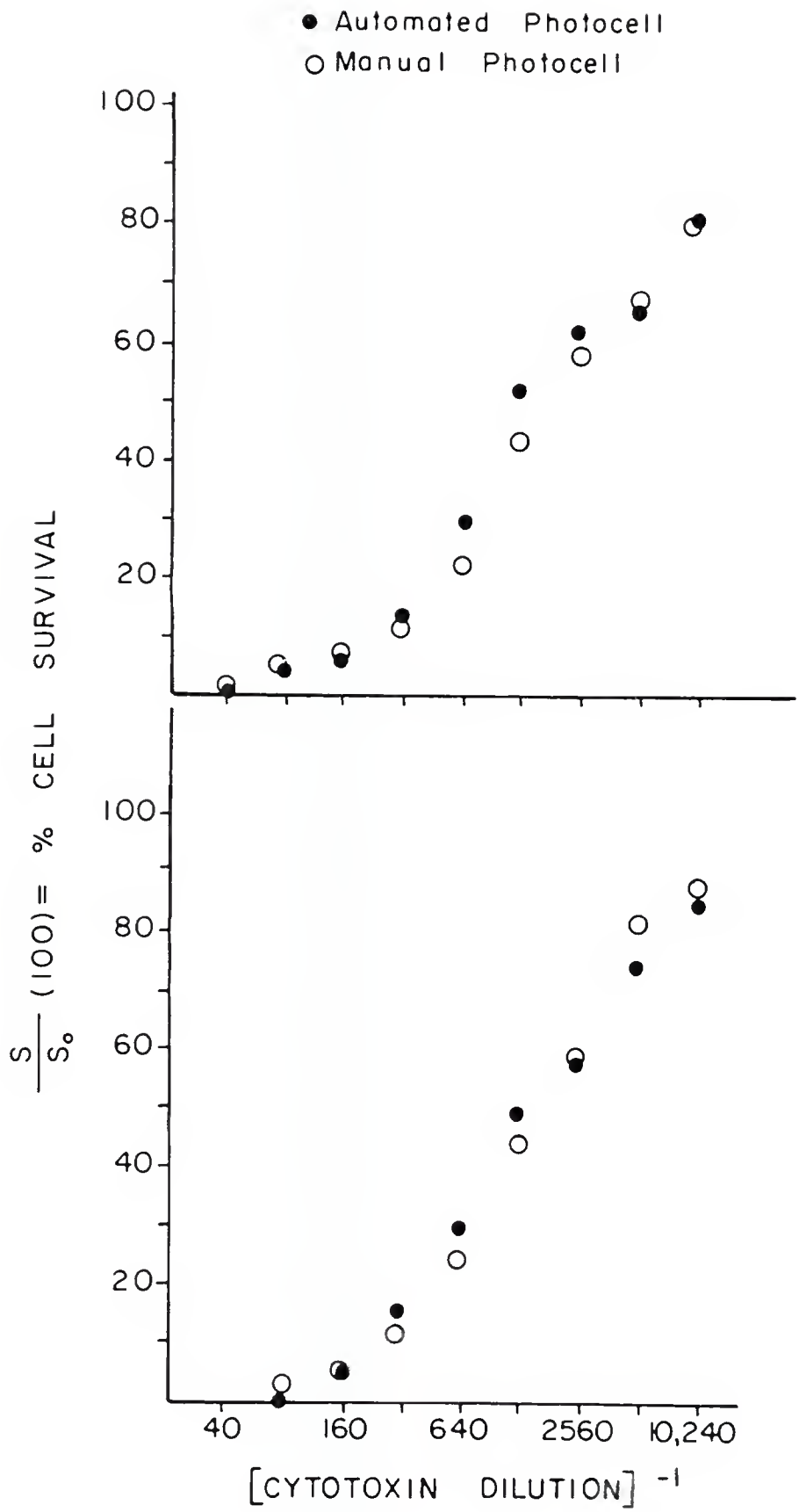
Plaque Assay

L-929 cells were grown to monolayers in plastic 24 well flat bottom tissue culture trays, plating 250,000 cells in 1 ml medium. After the monolayers were pretreated with actinomycin D (2 µg/ml) for 2 to 3 hours, they were washed twice with medium. Small numbers of well mixed effector cells (mouse PEC or rabbit PLC) in 2-fold increments were added carefully to washed and drained monolayers of targets in a final volume of 0.2 ml medium. Control target monolayers received only 0.2 ml medium. No agar

Figure 1. Comparison of automated and manual photocell in the photometric cytotoxicity assay.

Each panel (upper and lower) represents titration of a single sample by each of the photocells. Each point represents percent target cell survival of a single culture well.

S = number of cells surviving in cultures, S_0 = number of cells plated.


● Automated Photocell
○ Manual Photocell
100
80
60
40
20
100
80
60
40
20
$\frac{S}{S_o}$ (100) = % CELL SURVIVAL
40
160
640
2560
10,240
[CYTOTOXIN DILUTION] $^{-1}$

or methyl cellulose overlays were used. LPS was added at a final concentration of 10 µg/ml to PEC and PLC cultures. Plates were cultured undisturbed at 37°C for 18 to 20 hours. Supernatant contents were then decanted and the plates were stained for 10 minutes with crystal violet, washed with water and allowed to dry. In all cases, at least 95% of the effector cells plated were single cells and the number plated was based on number found to be viable (always >95%).

Cr^{51} Release Assay

L-929 cells were suspended into two 1 ml aliquots of 2×10^6 cells. One aliquot was treated with actinomycin D for 1 hour. At that time, 100 µCi sodium^{51} chromate (^{51}Cr; ICN Chemical, Waltham, MA) was added to each aliquot and the cells gently mixed by shaking. After 1 hour at 37°C the target cells were washed 5 times by repeated centrifugation and resuspension in fresh medium (MEM with 10% fetal bovine serum, gentamicin and HEPES buffer) to remove unincorporated ^{51}Cr and from one of the aliquots, actinomycin D. The concentration of the suspension was adjusted with medium to 3×10^4 cells/0.1 ml. $B16C_3$ cells were labelled in a similar way only in the absence of actinomycin D.

These labelled targets were used to determine cytotoxicity of mouse PEC (L-929) or CT generated by rabbit macrophage cultures (L-929, $B16C_3$).

Mouse PEC were seeded into 96 well flat bottom plastic tissue culture plates in MEM with 15% fetal bovine serum, gentamicin, and HEPES buffer at 3×10^5 cells in 0.1 ml volumes. Plates were centrifuged at 200 g for 30 seconds to sediment the cells to well bottoms. After 2 hours at 37°C in a humidified air atmosphere containing 5% CO_2, the nonadherent cells were removed by decanting the supernatants and washing

the adherent PEC monolayers twice gently with warm medium. The great majority of these adherent PEC, greater than 90%, had macrophage morphology as revealed by staining with Giemsa's blood stain and could phagocytize latex particles. After the second wash, 0.1 ml MEM with 10% fetal bovine serum, HEPES buffer and gentamicin was added to each well. In an additional 0.1 ml of similar medium, ^{51}Cr-labelled targets were added. LPS was then added. Plates were centrifuged at 200 g for 30 seconds to sediment target cells upon effector monolayers. Plates were incubated at 37°C, in a humidified air atmosphere containing 5% CO_2. At appropriate times for harvesting the supernatants, 0.05 ml of medium was added to each well to insure an even distribution of released ^{51}Cr throughout the cultures. Cr^{51} labelled targets (L-929 or $B16C_3$ cells) were added to microtiter wells along with TNSCT, PLCCT, or BMCCT. Again, at harvest times, 0.05 ml of medium was added to wells to evenly distribute released Cr^{51}.

For harvest, aliquots of 0.1 ml were removed and counted in a gamma counter (Gamma 300 Radiation Counter, Beckman Instruments, Inc., Fullerton, CA). For each time period, the percent specific ^{51}Cr released was determined by use of the following formula: percent release = $[(E-S \div (T-S)] \times 100$, where E = counts released from labelled targets in test cultures containing effectors and targets, S = spontaneous counts released from labelled targets cultured alone and T = total releasable counts from labelled targets, as determined by maximum lysis of targets with 1% dodecyl sodium sulfate. Appropriate volume correction factors were employed to calculate counts released in each case.

Column Chromatography

Gel Filtration

All gel filtration chromatography was performed using a single, calibrated column of Sephacryl, S-200 (Pharmacia Fine Chemicals, Piscataway, NJ) of gel bed dimensions 88 x 2.5 cm. Flow rate was 21 ml/hr using phosphate buffered saline. Samples were concentrated by pressure dialysis in an Amicon Diaflo ultrafiltration unit (Amicon Corp., Lexington, MA) prior to loading 1 ml samples onto the column. Fractions (2 ml) were collected and assayed for cytotoxicity on L-929 cells.

Ion Exchange Chromatography

Fractions with cytotoxicity for L-929 cells from the Sephacryl S-200 column were pooled and concentrated by pressure dialysis and loaded onto a column of DEAE-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) previously equilibrated with phosphate buffered saline (pH 7.2, 0.15 M). The column was washed with 2 column volumes of starting buffer to remove unbound proteins. A linear salt gradient (0.15 to 0.60 M) was applied to the column and fractions (4 ml) collected and assayed for cytotoxicity. Flow rate was 20 ml/hr.

All chromatography was performed at 4°C.

Effect of Trypsin Inhibitors, Phenylmethyl Sulfonyl-fluoride, Chloroquine and o-Phenanthrolene on Rabbit Macrophage Cytotoxin Activities

Various drugs were tested in the actinomycin D cytotoxicity assay to determine their effects on CT activity. The following were tested: soybean trypsin inhibitor (SBTI, Worthington Biochemical Corp., Freehold, NJ), bovine pancreatic trypsin inhibitor (BPTI, Sigma Chemical Co., St. Louis, MO), at 100 µg/ml each; phenylmethyl sulfonylfluoride (PMSF) at 10^{-4}M; chloroquine at 10^{-4}M, and o-phenanthrolene at 10^{-5}M (last 3 drugs from Sigma Chemical Co., St. Louis, MO).

Effects of Prostaglandin E_2 on Rabbit Macrophage Cytotoxin Production

Prostaglandin E_2 (PGE_2, Sigma Chemical Co., St. Louis, MO) was dissolved in ethanol and diluted in serum-free medium immediately before addition to duplicate PLC cultures in serum-free medium. PGE_2 was added at 10^{-6}M, 6 hours after initiation of the cultures with LPS (10 µg/ml). Controls received no PGE_2. PGE_2 antiserum (0.1 ml of lyophilized stock reconstituted with 2.5 ml buffer, Sigma Chemical Co., St. Louis, MO) was added to another set of cultures at the same time as LPS. Controls for this received no PGE_2 antiserum. These latter experiments were performed in the conventional MEM containing fetal bovine serum.

Effects of Actinomycin D on Rabbit Pulmonary Lavage Cell Cytotoxin Production

Actinomycin D was added at 1 µg/ml at the same time and at various times after addition of LPS to PLC cultures. Cultures were incubated for 30 hours, supernatants harvested and assayed.

Effect of Tunicamycin on Molecular Weight of Rabbit Pulmonary Lavage Cell Cytotoxin

Tunicamycin (Sigma Chemical Co., St. Louis, MO) was added to test PLC cultures at 0.5 and 5 µg/ml both 2 hours before and at the same time as LPS addition. Cultures were incubated for 30 hours at 37°C at which time, supernatants were harvested and titrated for cytotoxic activities. Cytotoxin was subjected to gel filtration chromatography on Sephacryl S-200 (previously described).

Freeze-Thawing of Activated Rabbit Pulmonary Lavage Cells

Pulmonary lavage cells were cultured in the presence of LPS (10 µg/ml) and at various times, replicate cultures were terminated. From some, supernatants were harvested and titered as previously described.

Other cultures were subjected to five freeze-thaw cycles in a dry ice-ethanol bath. After freeze-thawing, the culture contents were centrifuged and both supernatants and pellets titrated for cytotoxicity.

Susceptibility of Tumor Necrosis Serum Cytotoxin Resistant L-929 Cells to Rabbit Macrophage Cytotoxins and Pulmonary Lavage Cells

Matthews (1978) has demonstrated that it is possible to select for TNSCT-resistant L-929 cells by growing L-929 cells in the presence of small amounts of TNSCT. Using a lot of TNS which had an S_{50} titer (See Cytotoxicity Assays, Photometric Assay) in the actinomycin D cytotoxicity photometric assay of approximately 8000 units, L-929 resistant cells were selected for by growing cells in the presence of a 1:500 dilution of this CT. After about 2 weeks, during which time dead cells were decanted and viable cells were refed twice a week with fresh medium containing TNS, resistant cells were trypsinized, washed and plated into microtiter wells (10,000/well). Conventional L-929 cells were plated into another set of wells. After allowing cells to adhere overnight in conventional medium, TNSCT, PLCCT, BMCCT, or PLC (100 or 1000 cells) were added to test wells (control wells received only medium). In those wells containing PLC, LPS (10 µg/ml) was added. After 48 hours, plates were stained and read as previously described.

Time Lapse Cinematography

L-929 cells were plated into 25 cm^2 tissue culture flasks and allowed to adhere and form a monolayer overnight. The cells were treated with actinomycin D (2 µg/ml) for 2 to 3 hours, then washed extensively. Rabbit PLC were added in small numbers (10×10^3 to 50×10^3) to the washed monolayers in 3 ml medium at which time, LPS was added (10 µg/ml). Plates were allowed to equilibrate for an hour in a lucite incubator chamber mounted on an inverted phase contrast microscope

connected to a time lapse motion picture camera. A temperature of 37° to 38°C was maintained within the lucite chamber by a heated air curtain with a thermostat sensing device. An appropriate field was located (containing as few macrophages as possible). The field of view was photographed through a green filter. Photography was generally commenced 4 to 6 hours after addition of effector cells and LPS. Exposure rate for the first 2 to 3 hours of filming was 1 frame/15 seconds. Generally after that time, exposure rate was increased to 1 frame/8 seconds. Several films were made at magnifications of 150 and 200 X. Films were viewed at 24 frames/second.

RESULTS

Percent Macrophages in Rabbit Effector Cell Populations

Table I illustrates the percentage of cells that are macrophages from three sources, in several rabbits, as judged by morphological and phagocytic criteria. Pulmonary lavage cells represented a population made up almost exclusively of macrophages (90-95%). Blood mononuclear cells were 12-20% macrophages while bone marrow cells contained no more than 0.1-0.5% macrophages.

Increasing the Macrophage Content of Bone Marrow Cell Cultures

It is obvious from Table I that bone marrow cells provided only negligible amounts of macrophages. Lotem and Sachs (1979) have demonstrated that the phorbol ester, TPA, can cause both mouse and human myeloid leukemic cells to differentiate into cells with varying degrees of several macrophage characteristics (adherence to surfaces, Fc and C_3 receptors, phagocytic capability, lysozyme activity, and macrophage morphology).

Treatment of rabbit bone marrow cell cultures with TPA (1 μg/ml) resulted in adherence of many cells to the culture dish by 30 minutes. By 5 hours, over 90% of the cells were adherent (Table II). In addition, about one-third of the adherent cells had macrophage morphology and phagocytized latex beads. Twenty percent stained positive for peroxidase. (Later studies indicated that 0.1 μg/ml TPA was adequate to cause similar differentiation changes in rabbit bone marrow cells.)

TABLE I

RABBIT CELL POPULATIONS UTILIZED AS MACROPHAGE SOURCES

Source	Percent Macrophages[a]
Pulmonary Lavage Cells	90 - 95
Blood Mononuclear Cells	12 - 20
Bone Marrow Cells	<1

[a]Macrophage morphology (Giemsa's blood stain) and phagocytosis of latex beads.

TABLE II

PERCENT RABBIT BONE MARROW CELLS WITH MACROPHAGE PROPERTIES BEFORE AND AFTER TPA TREATMENT

Macrophage Property	Percent of Cells Before	Percent of Cells After TPA[a]
Adherence	<1	92[b]
Morphology	<1	32[c]
Phagocytosis (latex beads)	NT[d]	35[c]
Peroxidase positive	NT	20[c]

[a]5-28 hours after adding TPA at 1 μg/ml.

[b]Percent of total bone marrow cells added to culture.

[c]Percent of total adherent bone marrow cells.

[d]Not tested.

Considering the variable degrees of each property present, this process can be envisioned as a spectrum of variable differentiation in the direction of the mature macrophage state.

In Vitro Cytotoxin Production by Rabbit Macrophages

Requirement for Activating Agent

Matthews (1978) has reported on the apparently consistent, spontaneous production of CT by rabbit BLM cultures in the absence of known added activating agent (e.g., LPS). These studies did not determine the possible presence of contaminating LPS in the culture medium. This is often a problem in studying macrophages and can lead to serious misinterpretation of experimental results (Fumarola, 1981). Figure 2 demonstrates the results from experiments employing rabbit PLC and BLM cultures, where no attention was given to the possibility of contaminating LPS. Depending upon the time cell supernatants were harvested, the cytotoxin titers in the cultures to which LPS was added (1 μg/ml) were consistently greater than titers generated in cultures to which no LPS was added (3 to 20 times greater for BLM, 10 to 50 times greater for PLC, replicate cultures varied from mean values by ± 50%).

Using the Limulus amebocyte lysate test (Levin et al., 1970), it was determined that the culture system used for the experiments demonstrated in Figure 2 contained LPS (>0.025 ng/ml). Table III illustrates the various materials tested. The recycled glassware used to store prepared media tested positive for LPS. This was probably contaminated with the LPS-positive bovine serum lot (Gibco) also shown in Table III. Other than the glassware and Gibco bovine serum, only the trypsin stock concentrate (Microbiological Associates) and thioglycollate broth base powder (Difco) tested positive in the Limulus assay.

If no LPS was added to culture systems testing negative for LPS (<0.025 ng/ml) by the Limulus assay, rabbit PLC generally produced no CT

Figure 2. Rabbit macrophage cytotoxin production with and without added LPS.

Cytotoxin production by rabbit macrophage cultures in the presence (1 μg/ml) or absence of added LPS. Culture supernatants were harvested for assay at times indicated. Blood monocytes were cultured at 4×10^6/ml, pulmonary lavage cells at 1×10^6/ml.

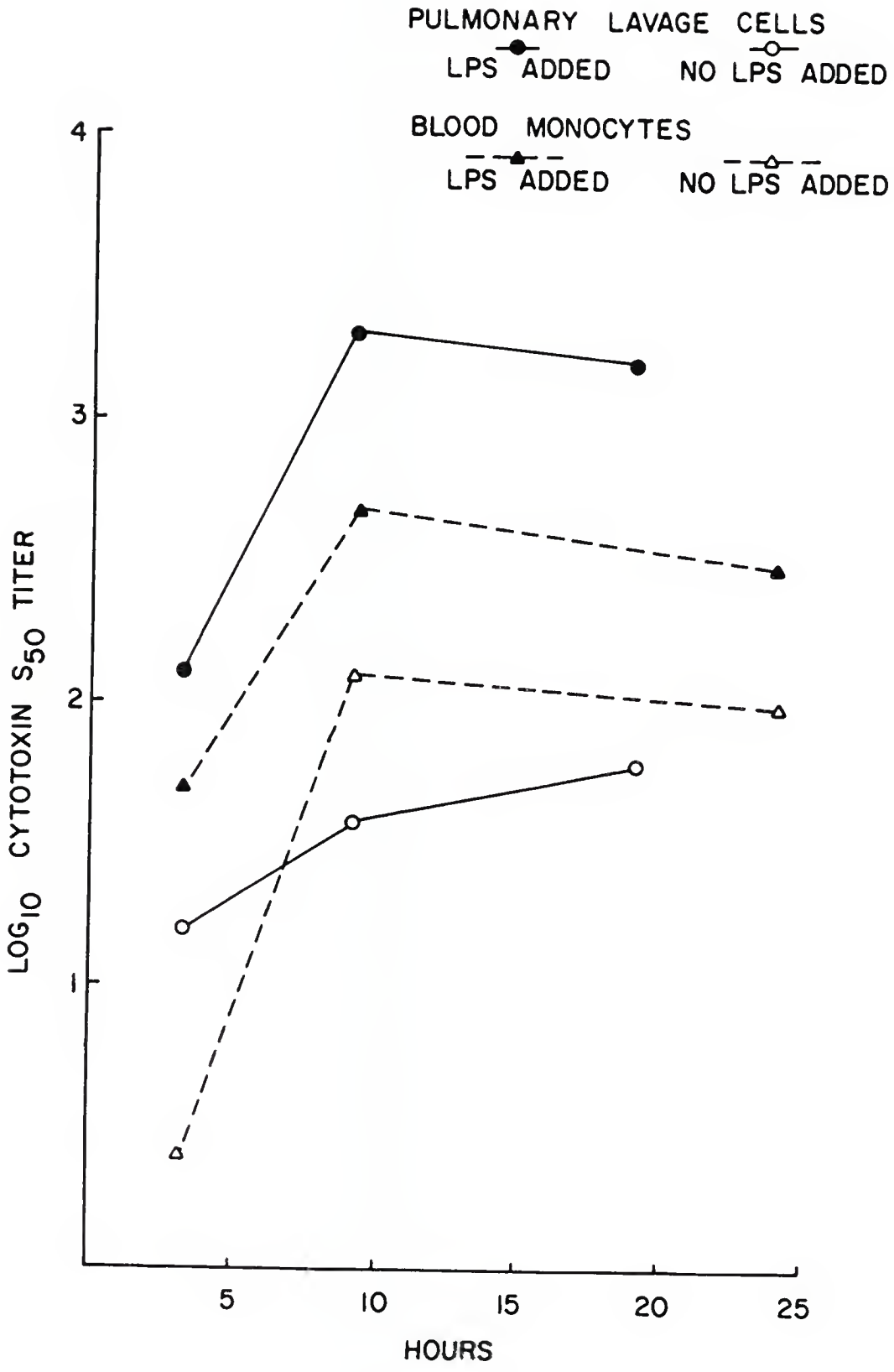

PULMONARY LAVAGE CELLS
LPS ADDED
NO LPS ADDED
BLOOD MONOCYTES
LPS ADDED
NO LPS ADDED
LOG10 CYTOTOXIN S50 TITER
4
3
2
1
5
10
15
20
25
HOURS

TABLE III

ITEMS TESTED WITH LIMULUS AMEBOCYTE LYSATE REAGENT

Item	Test Results[a]
Bovine calf serum	
Gibco; lot No. R290222	+
Sterile Systems, Inc., lot No. 400168	0
Fetal bovine serum	
K.C. Biol., lot No. 309447	0
Trypsin	
Stock concentrate, Microbiol. Assoc.	+
Crystalline, Sigma; bovine, porcine pancreas	0
Eagle's minimum essential medium, base concentrate	0
Glutamine, stock concentrate	0
Gentamicin, stock concentrate	0
HEPES buffer, stock powder	0
Deionized water from ion exchange tap	0
Thioglycollate broth, base powder, Difco	+
Reusable autoclaved laboratory glassware[b]	+

[a]Limulus lysate test results interpreted as follows: (+) = contains at least 0.025 ng/ml LPS; (0) = contains less than 0.025 ng/ml LPS.

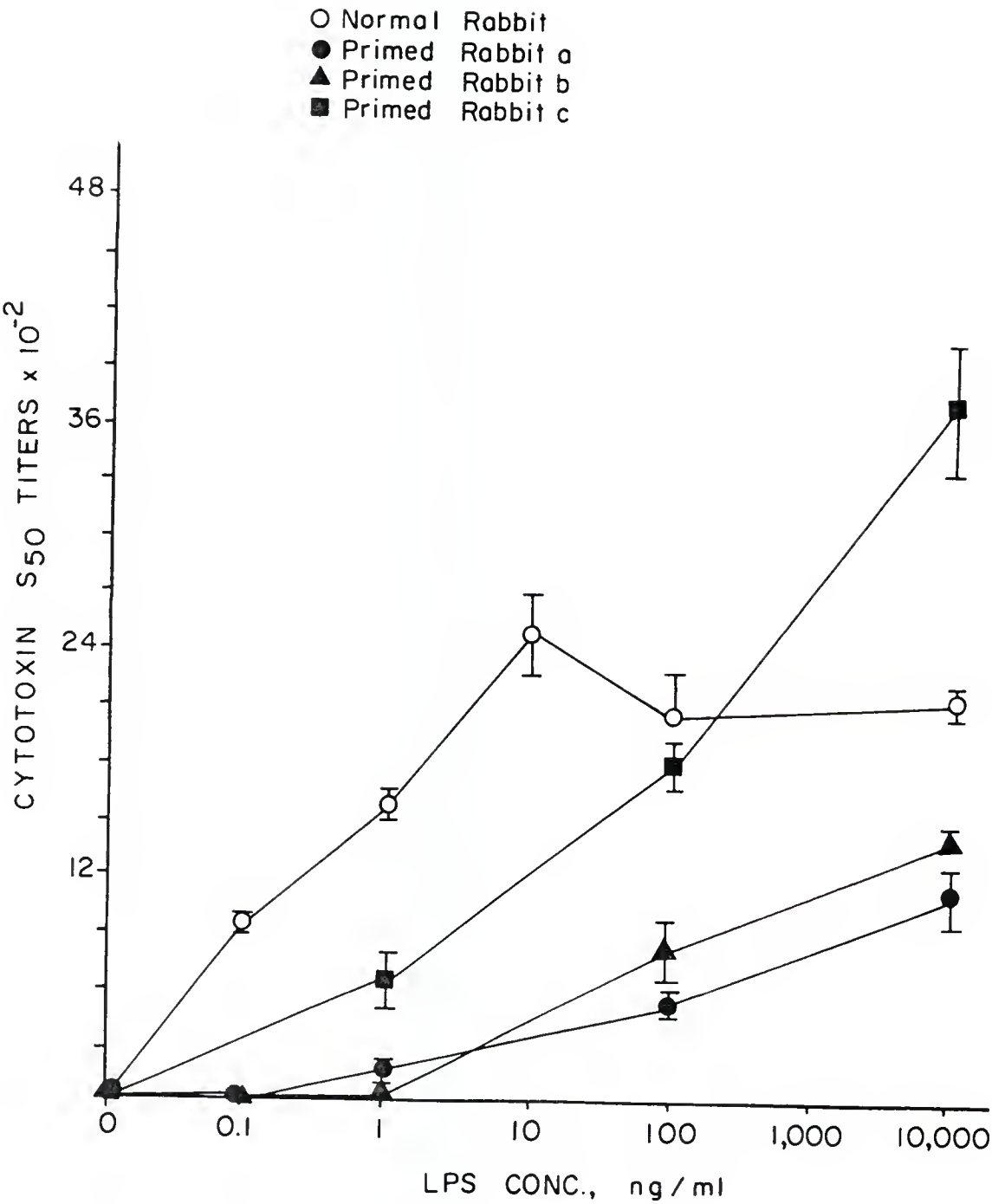
[b]Sterilized glassware rinsed with pyrogen free water; washings used for Limulus assay.

(Figure 3). However, one out of the ten rabbits sacrificed for these studies (only four rabbits are shown in Figure 3) had PLC that could produce detectable levels of CT in LPS-free cultures. Pasturella species was cultured from lung washings from this rabbit as well as from its PLC cultures. The presence of a Gram negative infection in the lungs probably resulted in in vivo activation of PLC. Despite this problem, the great majority (90%) of the rabbits utilized provided PLC that would not produce CT unless LPS was added to the culture (Figure 3). As little as 0.1 to 1 ng/ml could induce CT production. As a precaution, PLC removed from all rabbits to be utilized in other experiments (other than to merely generate CT for physicochemical studies) were tested in LPS-free culture systems for their ability to produce CT in the absence of added LPS. This insured that CT was not produced unless LPS was added to the culture system, allowing control over PLC activation.

As previously mentioned, many agents such as BCG (Old et al., 1960), Corynebacterium parvum (Halpern et al., 1974), etc., can cause a generalized macrophage hyperplasia in vivo. Leake and Myrvik (1968) have demonstrated this locally in the lungs of rabbits that were injected intravenously with BCG in mineral oil. They reported up to a 10-fold increase in the number of PLC obtained from rabbits injected intravenously over 1 week before sacrifice. They also reported qualitative changes in the PLC (an increase in cell lysozyme content as well as ultrastructural changes). Figure 3 shows that PLC from rabbits injected intravenously with complete Freund's adjuvant 2 to 3 weeks previously, produced no CT in the absence of added LPS and variable amounts of CT per 1×10^6 cells cultured in the presence of different levels of LPS. In addition, the normal rabbit shown (not administered Freund's adjuvant) produced significantly greater levels of CT at lower doses of LPS. Other experiments employing single dose levels of LPS in cultures of PLC demonstrated variable and overlapping

Figure 3. Rabbit macrophage cytotoxin production in the absence and presence of varying levels of exogenous LPS.

Pulmonary lavage cells from normal rabbits, or rabbits primed with Freund's complete adjuvant 2 to 3 weeks previously, were cultured in the absence or presence of varying levels of LPS. Supernatants were harvested after 24 hours. Culture systems contained <0.025 ng/ml LPS prior to LPS addition.


O Normal Rabbit
● Primed Rabbit a
▲ Primed Rabbit b
■ Primed Rabbit c
CYTOTOXIN S50 TITERS x 10-2
48
36
24
12
0
0.1
1
10
100
1,000
10,000
LPS CONC., ng / ml

results for CT production by LPS-activated PLC from normal and primed rabbits (data not shown). From this, it appears that both primed and normal rabbit PLC required the presence of LPS to produce CT *in vitro*. In addition, CT production by each cell type occurred to a similarly variable extent. Primed rabbits yielded 4 to 10 times more PLC (up to 200 x 10^6 cells) than normal rabbits, 80 to 90% of which had macrophage morphology (data not shown). In addition, LPS added to PLC cultures from primed or normal rabbits in amounts exceeding 10 µg/ml produced no more CT than at 10 µg/ml LPS (data not shown).

As with PLC, TPA-pretreated and washed BMC would not produce CT in LPS-free cultures unless LPS was added. In addition, no CT was produced from bone marrow cells that were not pretreated with TPA although exposed to LPS (bone marrow contains <1% macrophages, see Table I). Optimum CT production resulted when BMC were pretreated with TPA (0.1 and 1 µg/ml yielded similar results) for a minimum of 7 hours, then washed extensively (4 to 6 times) prior to activation with LPS (data not shown). This demonstrates the prerequisites for production of significant levels of CT by BMC. These are differentiation of sufficient bone marrow precursor cells to macrophages and subsequent activation of these macrophages by LPS.

Taking advantage of the strong and efficient adherence of BMC induced by TPA, BMC were plated at varying densities to determine the most efficient plating density for CT production (Table IV). Cultures producing the most CT had cells plated at approximately 0.5 x 10^6 and 1.5 x $10^6/cm^2$. This range of plating densities yielded consistently high CT titers for PLC as well (data not shown).

It was important that the TPA-pretreated BMC were washed extensively prior to adding LPS. Initial experiments with BMC were performed by adding LPS to TPA treated BMC without washing out the TPA. Virtually no CT was

TABLE IV

RABBIT BONE MARROW CYTOTOXIN PRODUCTION: OPTIMUM PLATING DENSITY[a]

Number Cells x $10^6/cm^2$	S_{50} Titer[b]	S_{50} Titer[b] per 10 x 10^6 cells
8.80	2290	104
5.92	2500	169
2.96	2500	338
1.48	2089	564
0.54	933	691

[a]Cells were cultured in MEM with 10% fetal bovine serum and 1 µg/ml TPA for 12 hours. TPA was removed by washing cultures extensively and LPS (1 µg/ml) was added.

[b]30 hour titers.

produced by these cultures. In order to determine if TPA could have an inhibitory effect on the CT, TPA was added to a TNSCT assay (Table V). A linear relationship was found between the logarithm of the amount of TPA added to the assay and the decrease in the titer of TNSCT. Pretreating the target cells (L-929) used in the assay with 100 ng TPA for 18 hours and then washing out the TPA prior to adding TNSCT to the cultures resulted in less inhibition of TNSCT titer (21% instead of 36% where only 2.4 ng TPA was left in the assay with TNSCT). These results demonstrate that TPA exerts an inhibitory effect on CT activity in general.

Comparison of Cytotoxin Production Capabilities by Rabbit Macrophages Derived from the Different Sources

Rabbit PLC, BMC and BLM were cultured under conditions that optimized CT production for each of the cell types. BMC cultured in a fortified medium (see Materials and Methods) generated about 4 times more CT than if cultured in the standard medium used for PLC and BLM. This was done to determine which of the cell types had the greatest potential for generating highest CT titers. Based upon 1×10^6 cells cultured per ml of medium, PLC were most efficient under the tested conditions (Table VI, 20 to 140 times more CT than BMC or BLM produced). Also as previously seen regarding sensitivity to LPS, PLC demonstrated much variability (about 7-fold) when comparing different rabbits with each other. BMC demonstrated much less variability (about 2-fold).

The overall cell populations used varied greatly in their macrophage content (Tables I and II). In addition, the BMC demonstrated a broad range of cells with macrophage characteristics depending upon which property was being utilized to call a cell a macrophage. It was therefore necessary to normalize the different cell populations for their macrophage content to compare the ability of macrophages from these

TABLE V

EFFECT OF TPA ON TUMOR NECROSIS SERUM (TNS) TITER

Experiment	TPA(ng)	Percent Inhibition TNSCT Activity
TNS titrated in presence of TPA[a]	2.4	36
	9.75	48
	78	70
TNS titrated on TPA-pretreated and washed targets[b]	100	21

[a]TPA was added to the standard actinomycin D serum cytotoxin photometric assay (see Materials and Methods).

[b]L-929 cells were preincubated with TPA for 18 hours, then washed. Serum cytotoxin photometric assay was then performed.

TABLE VI

CYTOTOXIN PRODUCTION BY RABBIT MACROPHAGES FROM BLOOD MONONUCLEAR, TPA-PRETREATED BONE MARROW AND PULMONARY LAVAGE CELLS: COMPARISON OF TITERS PRODUCED PER 1 x 10^6 CELLS

Cell Type[a]	Rabbit	S_{50} Titer per 1 x 10^6 Cells
Pulmonary lavage[b]	1	14,000
	2	4,562 ± 526
	3	2,200 ± 100
TPA-pretreated bone marrow[c]	4	70 ± 4[d]
	5	77 ± 6[d]
	6	119 ± 0[d]
Blood monocytes[b]	1	79

[a]All cells derived from normal rabbits and cultured at 1 x 10^6/ml in medium containing 10% fetal bovine serum, LPS at 10 µg/ml and 3 x 10^{-2}M HEPES buffer. Plating densities, 0.5-1.5 x $10^6/cm^2$.

[b]Cultured in Eagle's minimum essential medium.

[c]Cultured in Eagle's minimum essential medium fortified with 2 X vitamins and essential amino acids, nonessential amino acids and sodium pyruvate.

[d]Corrected for loss of bone marrow cells that do not adhere following TPA-pretreatment (<10%).

different sources to generate CT. Considering the potential ranges of percent macrophages in each population as well as the extremes of CT titer produced by each of the cell types, the PLC were still most efficient at generating CT (see Table VII) on a basis of 1×10^6 macrophages/ml culture medium (4 to 250 times greater than BMC and 4 to 40 times greater than BLM).

Effects of Bovine and Fetal Bovine Serum on Rabbit Macrophage Cytotoxin Production

The cytotoxic capabilities of macrophages can be enhanced or inhibited by various serum components (Hibbs et al., 1977; Chapman and Hibbs, 1977). Mechanisms which could help explain this phenomenon may involve production or function of macrophage CT. PLC were cultured in the absence or presence of varying levels of bovine or fetal bovine serum (Figure 4). Under the conditions tested, it can be seen that PLC could generate CT in serum-free medium (PLC from two different rabbits generated 330 to 400 S_{50} CT units under the culture conditions described for Figure 4, panel a). Bovine serum was inhibitory to CT production at levels generally greater than 4%. Fetal bovine serum enhanced CT production by PLC up to 3-fold (at 10%). Figure 5 further illustrates that 10% fetal bovine serum was more effective than lower levels (1%) at potentiating LPS-induced PLCCT production. This was the case at 3 levels of LPS concentration. It can also be concluded that to the extent to which they were tested, LPS at 10 µg/ml with 10% fetal bovine serum were optimal together for potentiating the activation of PLC by LPS.

Time Kinetics of Macrophage Cytotoxin Production

Figure 6 demonstrates *in vitro* production of CT by LPS-activated PLC with respect to time. Generally, significant levels of CT appeared

TABLE VII

DATA FROM TABLE VI CONSIDERING PERCENT MACROPHAGE CONTENT OF EACH CELL TYPE

Cell Type	Percent Macrophages Possible Range	Theoretical Range of S_{50} Titer per 1×10^6 Macrophages
Pulmonary lavage	90 - 95	14,737 - 15,556 2,316 - 2,444
TPA-pretreated bone marrow	20 - 100[a]	70 - 350 119 - 595
Blood monocytes	12 - 20	395 - 658

[a]Considering all macrophage properties of TPA-pretreated and washed adherent bone marrow cells (e.g. peroxidase staining positive cells represent 20% of total adherent cell population. See Table I).

Figure 4. Effects of bovine and fetal bovine serum on pulmonary lavage cell cytotoxin production.

Rabbit pulmonary lavage cells were cultured for 24 hours in the absence or presence of various levels of bovine or fetal bovine serum. Experiments employing cells from 2 rabbits are shown: a. LPS present at 1 μg/ml; b. LPS present at 10 μg/ml. Control cultures contained no serum.

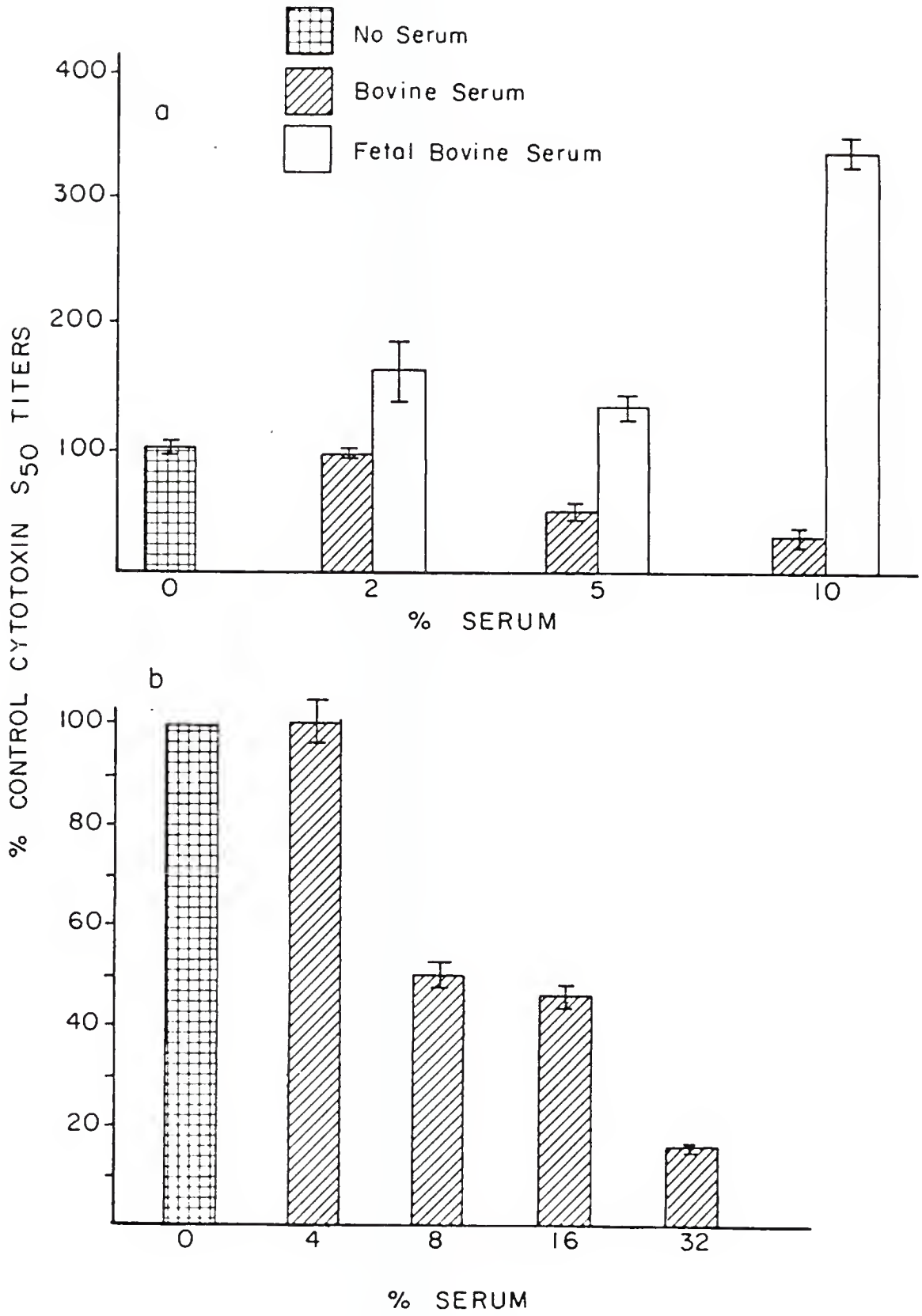

No Serum
Bovine Serum
Fetal Bovine Serum
a
400
300
200
100
% CONTROL CYTOTOXIN S_{50} TITERS
0
2
5
10
% SERUM
b
100
80
60
40
20
0
4
8
16
32
% SERUM

Figure 5. Comparison of effects of 1% and 10% fetal bovine serum on cytotoxin production by rabbit pulmonary lavage cell cultures containing various levels of LPS.

Supernatants were harvested from triplicate cultures that were incubated for 30 hours.

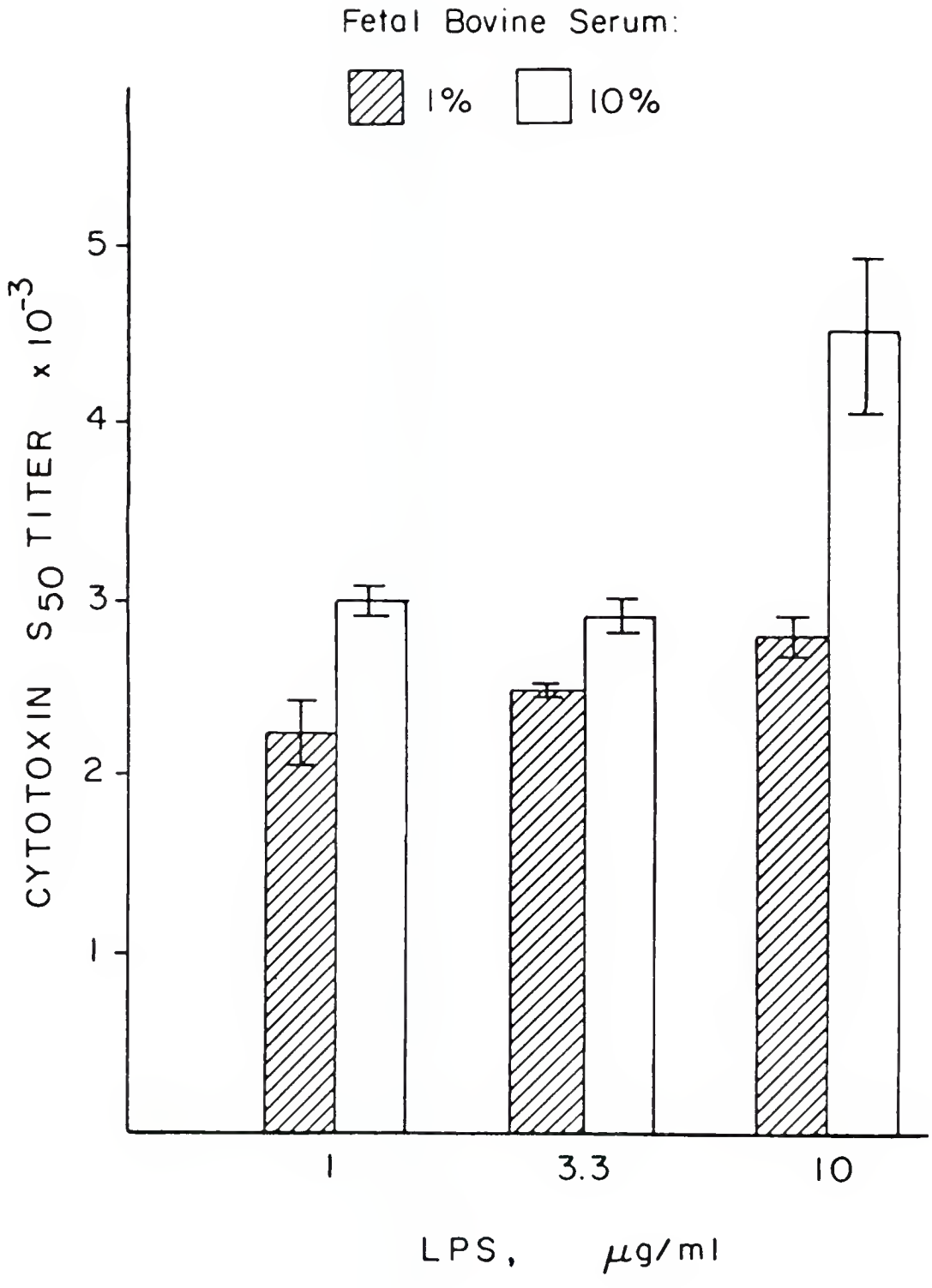
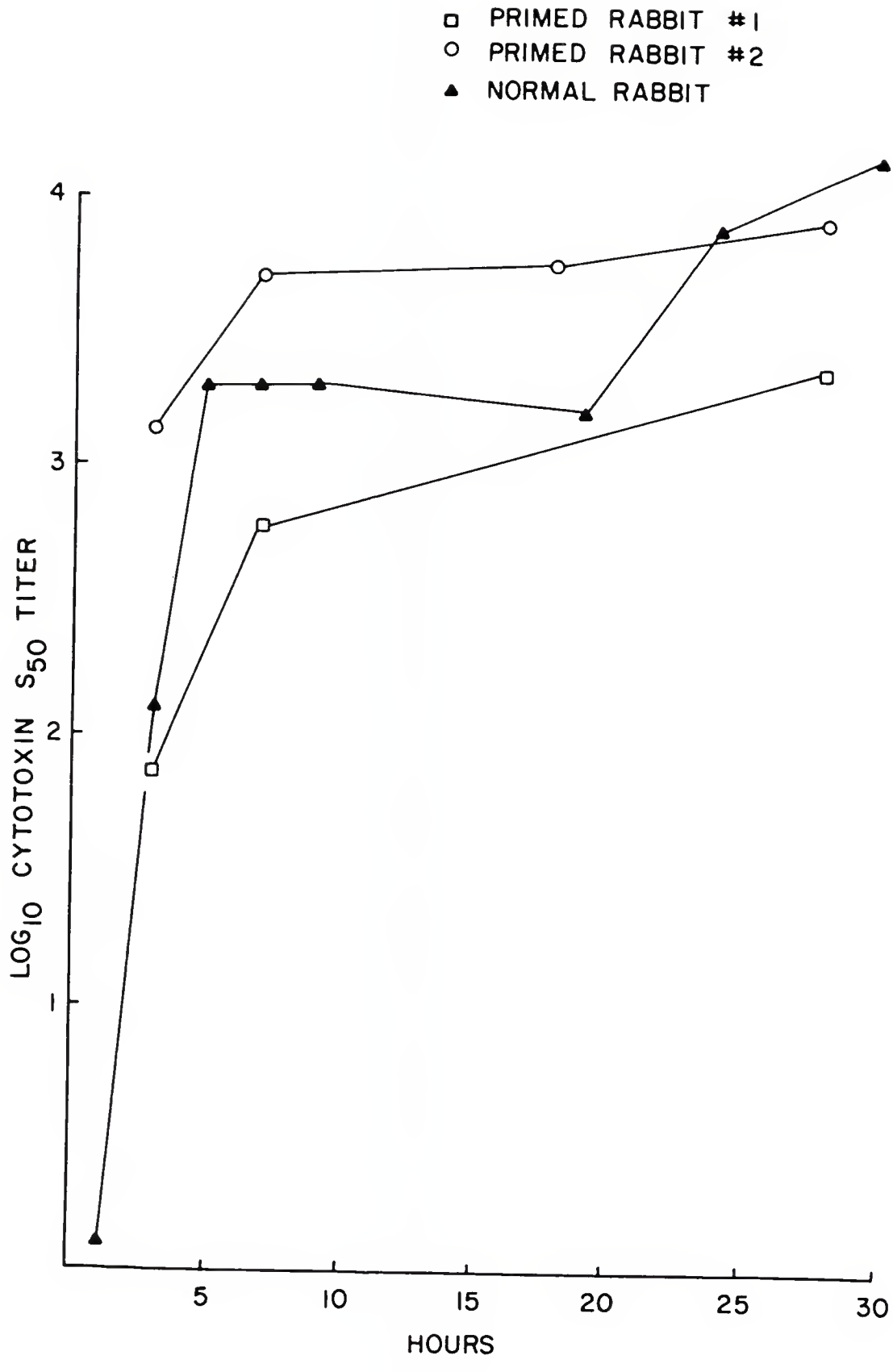

Fetal Bovine Serum:
1%
10%
CYTOTOXIN S50 TITER x 10-3
5
4
3
2
1
1
3.3
10
LPS, μg/ml

Figure 6. Time kinetics of rabbit pulmonary lavage cell cytotoxin production.

Pulmonary lavage cells from a normal rabbit or rabbits primed with Freund's complete adjuvant 2 to 3 weeks previously were cultured in the presence of LPS. Supernatants were harvested at times indicated.


PRIMED RABBIT #1
PRIMED RABBIT #2
NORMAL RABBIT
LOG10 CYTOTOXIN S50 TITER
4
3
2
1
5
10
15
20
25
30
HOURS

by 3 hours and titers increased significantly between 3 and 5 hours. Levels remained fairly constant at times tested between 5 to 7 and 18 hours and then increased significantly again after about 18 hours in culture. Variation of replicate cultures was mean ±20% for the Freund's adjuvant primed rabbits and mean ±50% for the normal rabbit. Figure 6 also demonstrates the variation among rabbits (normal and primed) in the CT titers generated by their PLC at any single given time. Other experiments involving culturing PLC from several normal and primed rabbits under identical conditions for the same time resulted in overlapping and similarly variable CT titers for both groups (data not shown).

Time kinetics of CT production by TPA-pretreated and washed, LPS-activated BMC are given in Figure 7. Detectable levels of CT (20 to 26 S_{50} units) appeared by 3 hours and increased through 14 hours. Levels measured at 14 and 30 hours were similar.

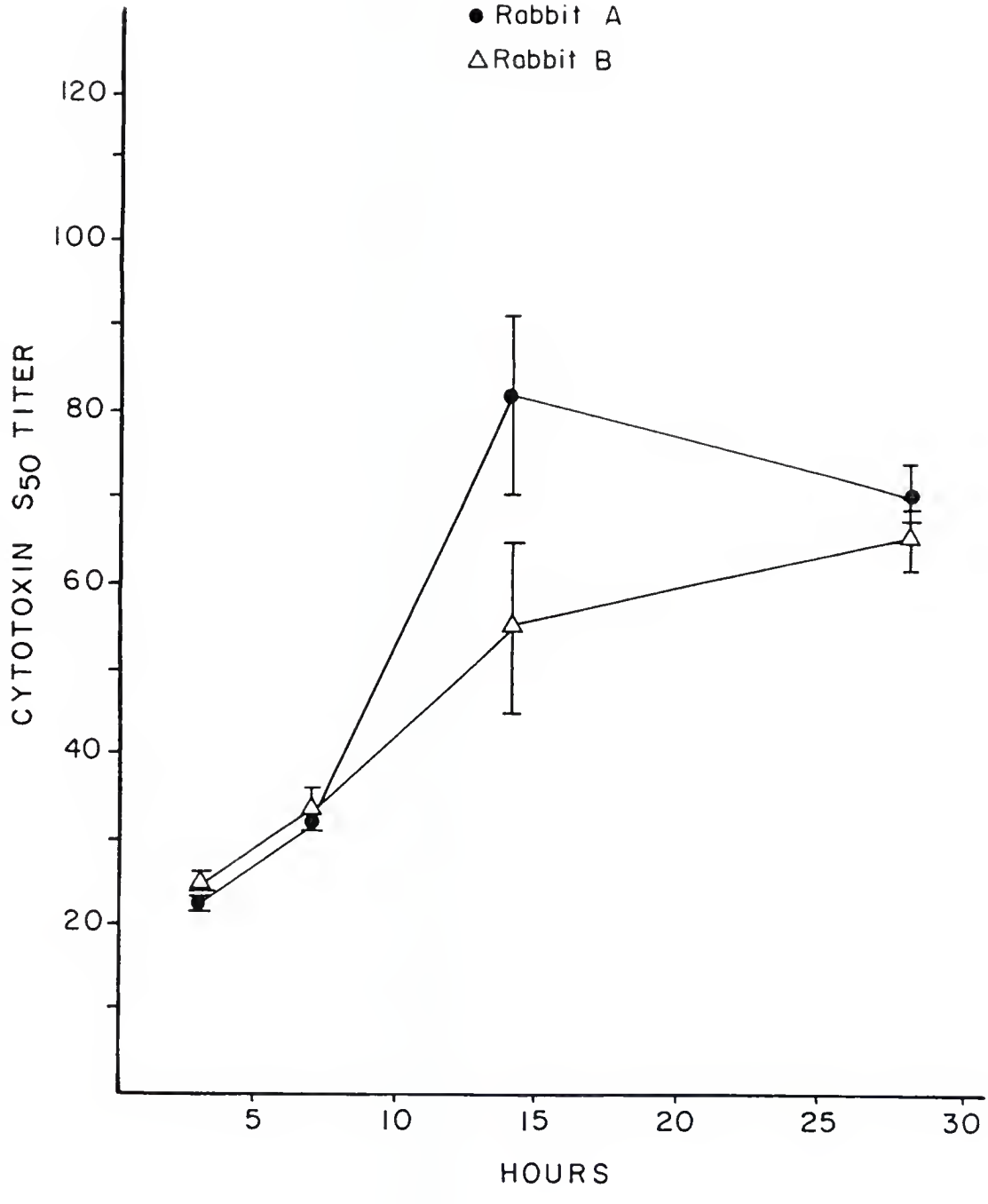
Time kinetics of CT production by LPS-activated BLM are included in Figure 2. Detectable levels of CT were present by 3 hours, increased between 3 and 7 hours and were similar at 7 and 24 hours. These observations are consistent with the data of Matthews (1978). From these observations, it is apparent that time kinetics of CT production by PLC, BMC and BLM are somewhat different after an initial production time during the first 3 hours. After 30 hours culture, essentially no CT was produced by PLC and BMC that were refed with fresh LPS-containing medium (data not shown).

Effects of Actinomycin D on Cytotoxin Production by Rabbit Pulmonary Lavage Cells

Cameron (1980) has described an actinomycin D-induced inhibition of cytotoxicity of human macrophages for tumor cells. The data presented

Figure 7. Time kinetics of cytotoxin production by TPA-pretreated and washed rabbit bone marrow cells.

Rabbit bone marrow cells were plated into 24-well tissue culture dishes and treated with TPA (0.1 µg/ml) overnight. After removing TPA from cultures (washed 5 times), fresh medium containing LPS (10 µg/ml) was added. Supernatants were harvested at various times.


● Rabbit A
△ Rabbit B
120
100
80
60
40
20
CYTOTOXIN S50 TITER
5
10
15
20
25
30
HOURS

in Figure 8 show that actinomycin D (1 µg/ml) consistently inhibited PLCCT production if added to cultures at the same time as LPS. (Inhibition of CT titers of test cultures was greater than 95% below those of control cultures to which no actinomycin D was added.) Delaying addition of actinomycin D resulted in a decrease of inhibition of CT titers. This varied considerably among rabbits, especially when comparing primed to normal rabbits. From the data of Figure 8, it is apparent that in no case was there active CT messenger RNA or active CT in PLC prior to the addition of LPS to the cultures. In addition, active CT messenger RNA was fully synthesized by 2 to 6 hours after LPS activation of primed PLC and about 3 hours after LPS-activation of normal PLC.

Effects of Prostaglandin E (PGE) and Prostaglandin Antiserum on Cytotoxin Production by Rabbit Pulmonary Lavage Cells

It has been demonstrated that PGE can be produced by macrophages in culture and can shut off macrophage cytotoxicity for tumor cells apparently after a period of macrophage activation (Taffet and Russell, 1981). Figure 9 demonstrates that PGE (10^{-6} M) added to PLC cultures 6 hours after LPS (10µg/ml) resulted in inhibition of CT production down to about 30% of control culture levels. In addition, adding PGE antiserum to cultures at the same time as LPS increased CT production by 50% over the control levels. These data are consistent with the conclusions of Taffet and Russell (1981) concerning regulation of macrophage cytotoxicity by endogenously produced PGE.

Disruption of Rabbit Pulmonary Lavage Cells at Various Times after LPS Activation: Failure to Demonstrate Significant Active Intracellular Cytotoxin Pool

Unanue and Kiely (1977) have demonstrated that a macrophage protein, mitogenic for thymocytes, accumulates within the macrophage in

Figure 8. Effects of actinomycin D on cytotoxin production by rabbit pulmonary lavage cells.

Actinomycin D (1µg/ml) was added to cultures of rabbit pulmonary lavage cells at the same time and various times after addition of LPS. Culture supernatants were harvested for assay after 30 hours. Panel a represents 2 primed rabbits, panel b, 2 normal rabbits.

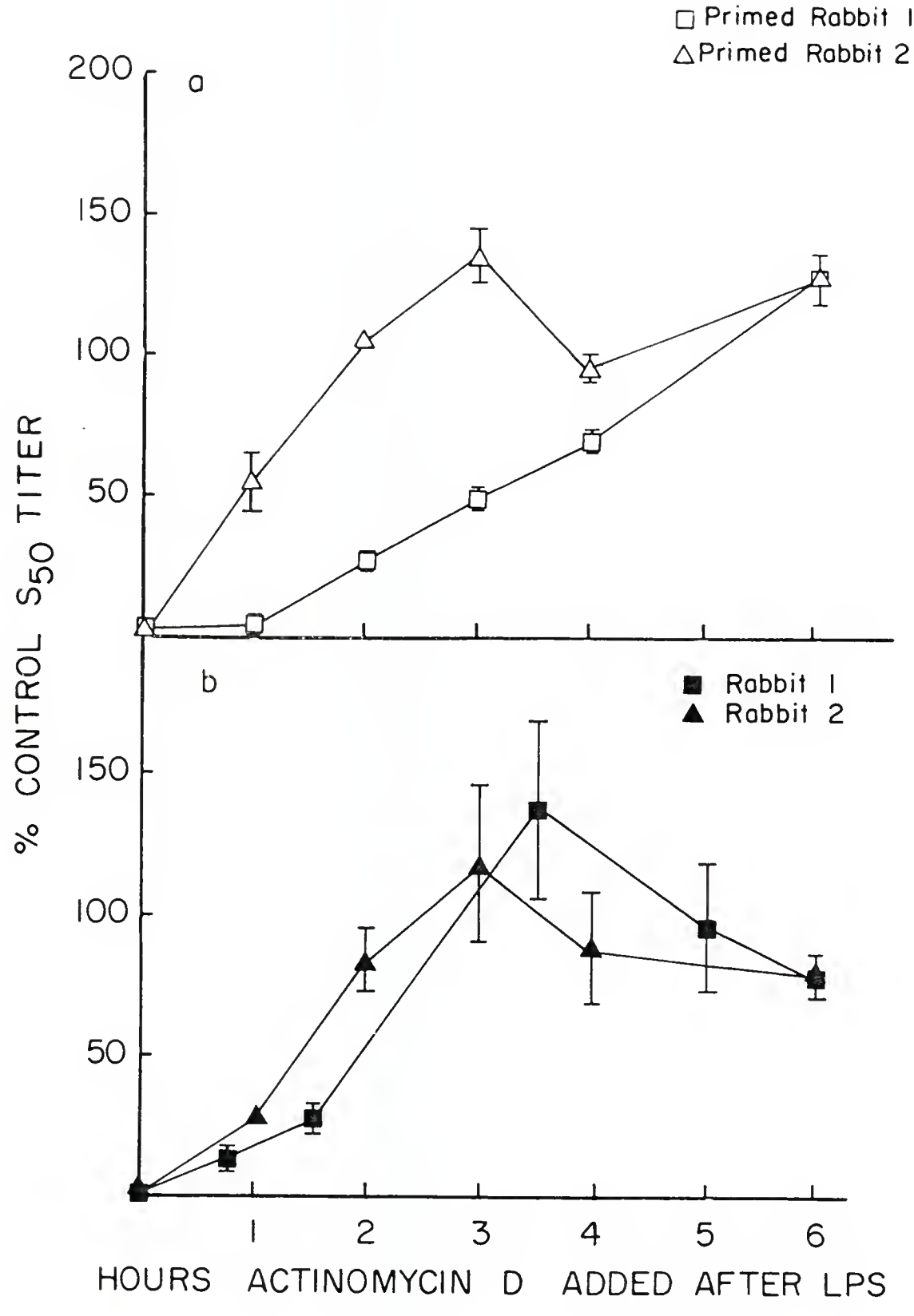
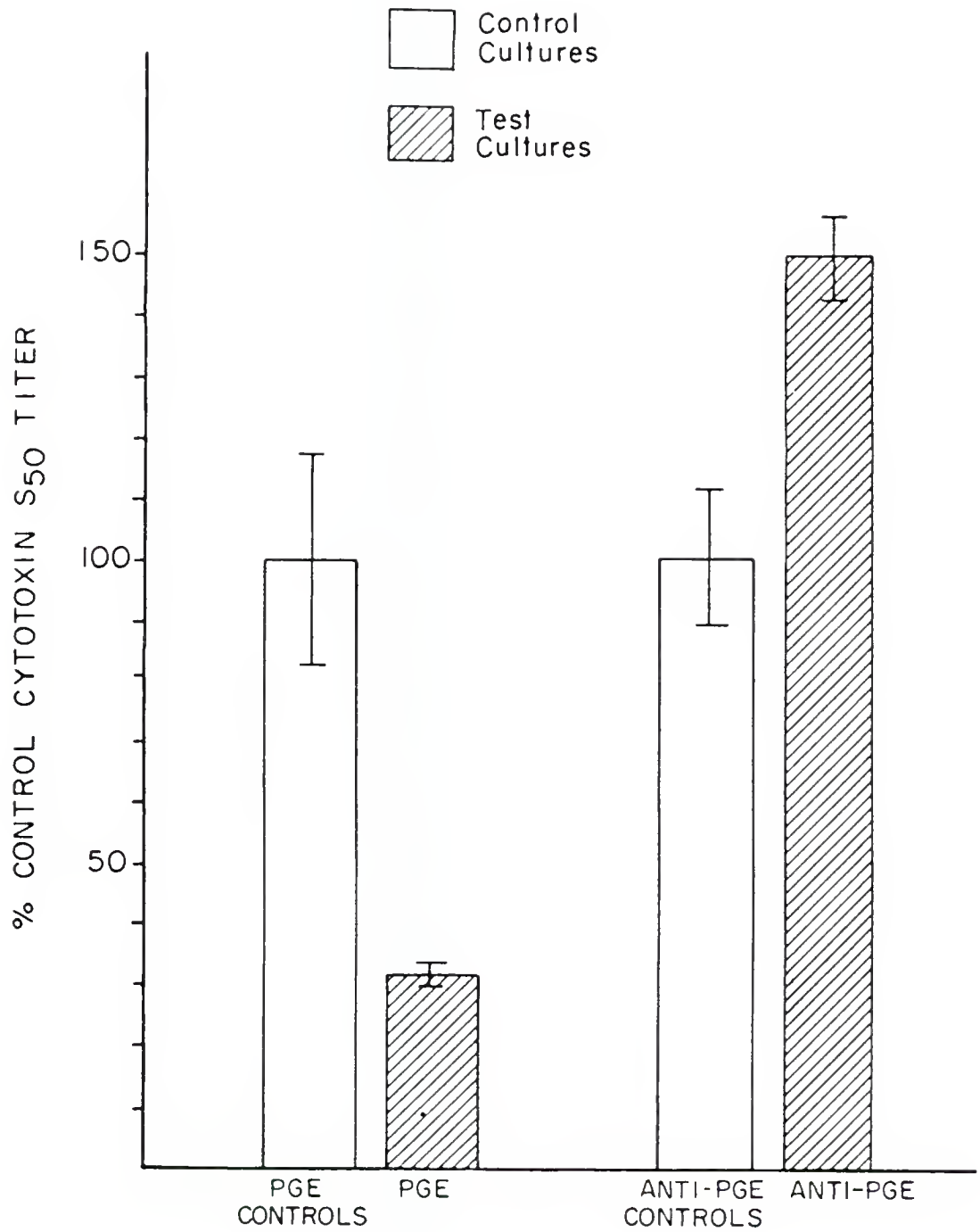

□ Primed Rabbit 1
△ Primed Rabbit 2
a
200
150
100
50
% CONTROL S_{50} TITER
b
■ Rabbit 1
▲ Rabbit 2
150
100
50
1
2
3
4
5
6
HOURS ACTINOMYCIN D ADDED AFTER LPS

Figure 9. Effects of prostaglandin E_2 and prostaglandin antiserum on cytotoxin production by rabbit pulmonary lavage cells.

Rabbit pulmonary lavage cells were cultured in the presence of LPS at 10µg/ml. To 1 set of cultures in serum-free FMEM, prostaglandin E_2 was added at 10^{-6} M. To another set of cultures in standard MEM supplemented with 10% fetal bovine serum, prostaglandin antiserum was added (see Materials and Methods). Cultures were incubated for 28 hours and supernatants harvested.


Control Cultures
Test Cultures
% CONTROL CYTOTOXIN S50 TITER
150
100
50
PGE CONTROLS
PGE
ANTI-PGE CONTROLS
ANTI-PGE

the form of an intracellular pool. Only a small portion of this pool may be secreted by the macrophage depending upon its state of activation. By repeated freeze-thawing of thioglycollate-stimulated mouse PEC, they could demonstrate higher levels of mitogenic protein within the cell than were secreted under certain conditions.

Rabbit PLC were activated by LPS and at various times after LPS addition (30 minutes, 1, 3, 7 and 24 hours), culture supernatants and freeze-thawed lysates were analyzed for CT. As previously, supernatant CT became measurable at 3 hours but at no time, except one (1 hour after addition of LPS) did freeze-thawed lysate contain measurable CT (data not shown). At 1 hour after LPS addition to cultures, only about 2 S_{50} units of CT appeared in freeze-thawed lysates while none appeared in the culture supernatant. Appropriate controls indicated total stability of PLCCT to repeated freeze-thawing in this culture system. In addition, assaying the freeze-thawed PLC (none viable by inability to exclude trypan blue dye) on L-929 cells in the presence of actinomycin D revealed no cytotoxicity. These studies indicate that no significant levels of active freeze-thaw-releasable CT were retained by the activated macrophage.

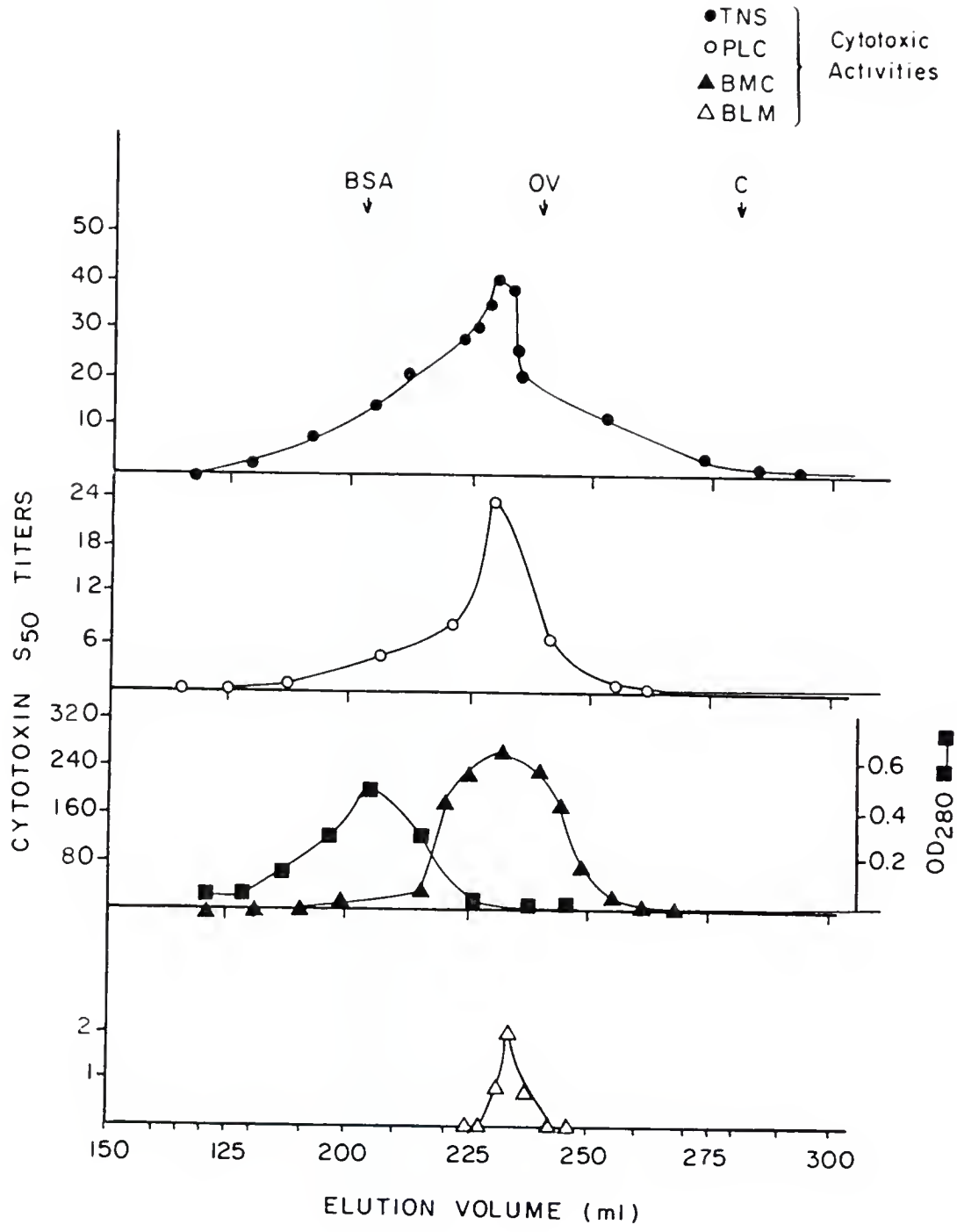
Physicochemical and Biological Properties of Rabbit Macrophage Cytotoxins and Tumor Necrosis Serum Cytotoxin

Gel Filtration Chromatography

All 4 of the cytotoxins were eluted through the same Sephacryl S-200 column. All eluted as single peaks. The elution volumes for all corresponded to a molecular weight of about 48,000 D (Figure 10).

Figure 10. Gel filtration of rabbit macrophage cytotoxins on Sephacryl S-200.

Macrophage culture supernatants were concentrated by pressure dialysis prior to gel filtration. The column was calibrated with standards of known molecular weight, ribonuclease (not shown), chymotrypsin (C), ovalbumin (OV), bovine serum albumin (BSA).


●TNS
○PLC
▲BMC
△BLM
Cytotoxic Activities
BSA
OV
C
CYTOTOXIN S_{50} TITERS
50
40
30
20
10
24
18
12
6
320
240
160
80
2
1
0.6
0.4
0.2
OD_{280}
150
125
200
225
250
275
300
ELUTION VOLUME (ml)

Ion Exchange Chromatography

The 4 cytotoxins were eluted through freshly poured DEAE-Sephadex columns with gel beds of approximately similar size. All eluted with the same linear salt gradient between 0.28 and 0.32 M NaCl (Figure 11).

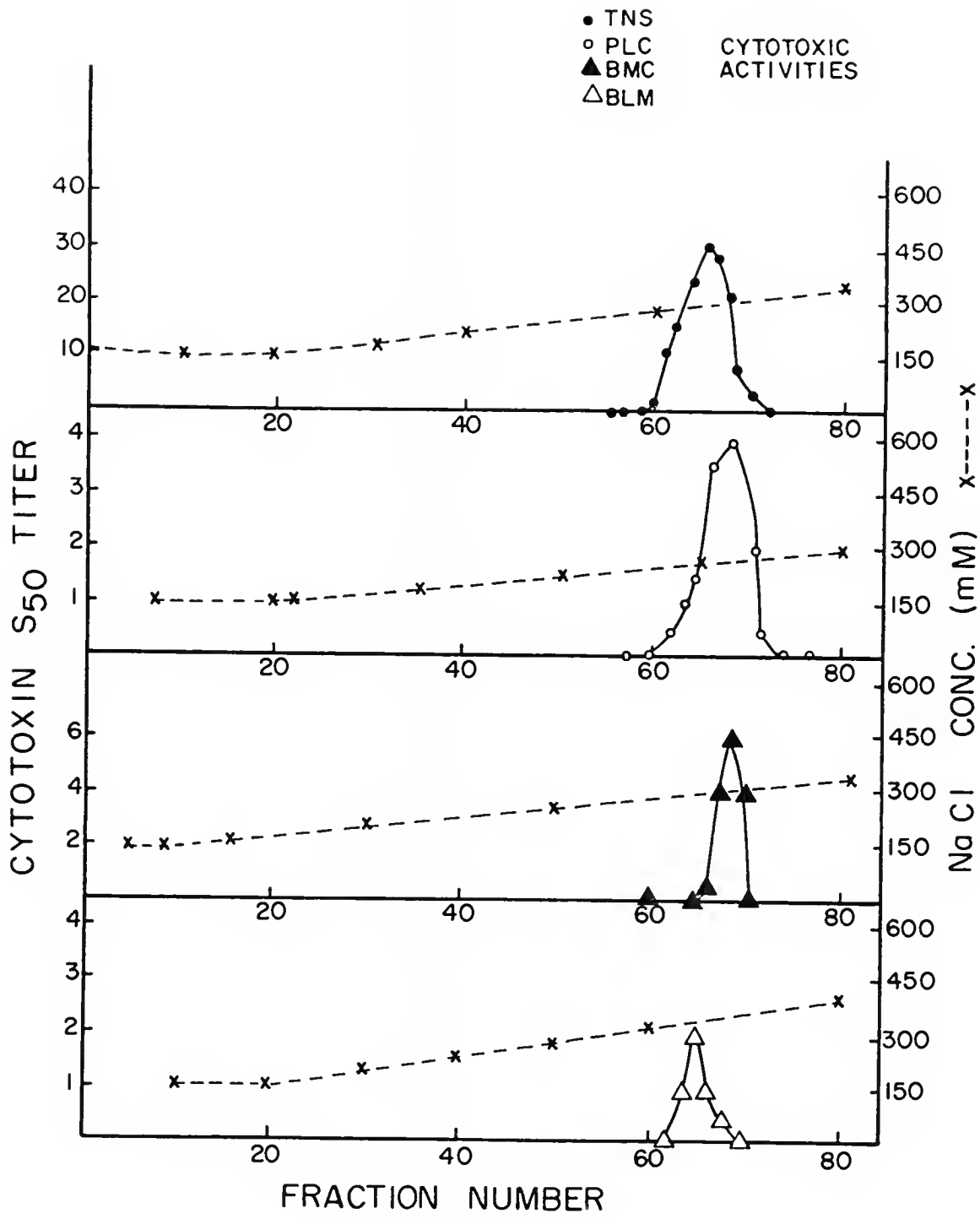
Effect of Tunicamycin on the Molecular Weight of Cytotoxin Generated by Rabbit Pulmonary Lavage Cells

Previous studies failed to demonstrate significant levels of exposed carbohydrate on rabbit TNSCT (Matthews et al., 1980; Ruff and Gifford, 1981). These studies drew their conclusions from the inability to bind TNSCT to a variety of sugars and plant lectins.

Tunicamycin has been shown to inhibit N-glycosylation of glycoproteins by blocking formation of N-acetylglucosamine-lipid intermediates (Hickman et al., 1977). This has been demonstrated for several types of glycoprotein including interferon (Fujisawa et al., 1978) and immunoglobulin (Hickman et al., 1977). Rabbit PLC were cultured with LPS both in the presence and absence of tunicamycin. Tunicamycin at 1 µg/ml had no effect on production of CT, but at 5 µg/ml, CT titers were decreased by 30% when compared to control cultures which contained no tunicamycin. As high as 5 µg/ml tunicamycin had no effect on the molecular weight of CT produced as determined by gel filtration chromatography on Sephacryl S-200 (data not shown). This demonstrates that CT has no covalently linked carbohydrate or has a covalently linked carbohydrate of very small molecular weight or could have a carbohydrate portion of substantial size that is covalently linked to the rest of the molecule by a tunicamycin resistant mechanism.

Figure 11. Ion exchange chromatography of rabbit macrophage cytotoxins on DEAE-Sephadex.

Rabbit TNS or pooled cytotoxin containing fractions from the Sephacryl S-200 column were concentrated by pressure dialysis and loaded in starting buffer. Unbound proteins were washed off the column, followed by elution with a linear salt gradient from 150 to 600 mM phosphate buffered saline.


• TNS
o PLC
▲BMC
△BLM
CYTOTOXIC ACTIVITIES
CYTOTOXIN S50 TITER
NaCl CONC. (mM) x----x
FRACTION NUMBER
40
30
20
10
4
3
2
1
6
4
2
4
3
2
1
600
450
300
150
20
40
60
80

Temperature Stability of Rabbit Macrophage Cytotoxins

Each of the cytotoxins were subjected to 56°C and 70°C in separate experiments (Figure 12). All four of the cytotoxins were stable to 56°C for 60 minutes. In addition, the titers of all were greater than the controls, which were maintained at 4°C during the course of the experiment. This may suggest a heat labile inhibitor of CT present. At 70°C, all cytotoxins demonstrated decay kinetics that were fairly similar and by 60 minutes, all lost about 80% of activity compared to controls.

Effect of Serine Protease Inhibitors and o-Phenanthrolene on Cytotoxin Activities

There is evidence in the murine system that serine proteases may play a role in macrophage mediated tumor cytolysis (Adams, 1980; Adams et al., 1980). Rabbit PLCCT was not inactivated by phenylmethyl sulfonyl fluoride (10^{-4}M), an inhibitor of serine proteases (data not shown). In addition, neither bovine pancreatic trypsin inhibitor nor soybean trypsin inhibitor inactivated PLCCT or BMCCT activity (data now shown). None of these enzyme inhibitors inactivated TNSCT either, consistent with the findings previously reported (Ruff and Gifford, 1981). On the other hand as this latter group has reported for TNSCT, o-phenanthrolene, a metal chelator had significant inhibitory effects on the macrophage CT (Table VIII).

Effect of Chloroquine on Rabbit Macrophage Cytotoxin Activities

Chloroquine is a drug which can apparently stabilize cell lysozomes (Lie and Schofield, 1973). Previous work with TNSCT (Ruff and Gifford, 1981) has demonstrated an inhibitory effect of this drug on cytotoxin activity in the actinomycin D assay. All 4 cytotoxins were tested in the standard actinomycin D assay with chloroquine (10^{-4}M) and all were inhibited (Table VIII).

Figure 12. Thermal stability of rabbit TNS cytotoxin and macrophage cytotoxins.

Rabbit macrophage culture supernatants and TNS diluted with culture medium were exposed to 56°C for 1 hour or 70°C for 20, 40 or 60 minutes. Control TNS and culture supernatants were maintained at 4°C for 1 hour.

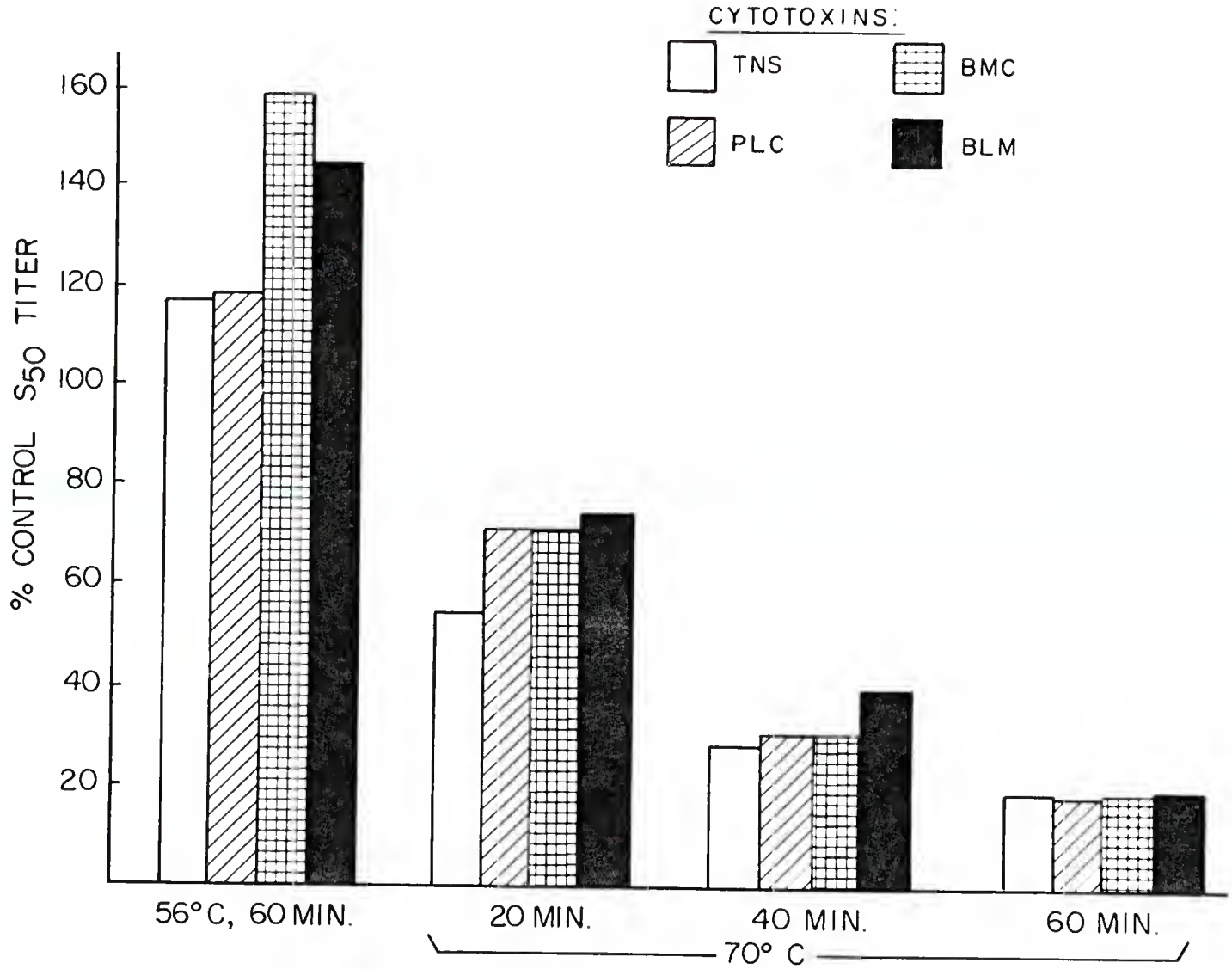

CYTOTOXINS:
TNS
BMC
PLC
BLM
% CONTROL S_{50} TITER
160
140
120
100
80
60
40
20
56° C, 60 MIN.
20 MIN.
40 MIN.
60 MIN.
70° C

TABLE VIII

EFFECTS OF CHLOROQUINE AND o-PHENANTHROLENE ON CYTOTOXIN ACTIVITY

Drug	Percent of Control S_{50} Titer			
	PLC[a]	BMC[b]	BLM[c]	TNS[d]
Chloroquine, 10^{-4}M	55	38	37	31
o-Phenanthrolene, 10^{-5}M	46	48	35	50

Cytotoxins from:

[a]PLC, pulmonary lavage cells

[b]BMC, TPA-pretreated bone marrow cells

[c]BLM, blood monocytes

[d]TNS, tumor necrosis serum

Effects of High Arginine Levels on Rabbit Macrophage Cytotoxin Activities

Many reports indicate that arginase plays some role in macrophage mediated tumor cytotoxicity (Currie and Basham, 1975, 1978; Currie, 1978; Chen and Broome, 1980). These same reports indicate inhibition of macrophage merdiated tumor cytotoxicity by adding excess arginine to the medium. Arginine added to our cytotoxin assay at 2×10^{-2}M (5 to 10 times the level employed by these latter investigators) did not inhibit cytotoxicity of any of the CT (data not shown). From this, we conclude that none of our cytotoxins is arginase.

Relative Titers of Rabbit Macrophage Cytotoxins in the Presence and Absence of Actinomycin D in the Cytotoxicity Assay

Ostrove and Gifford (1979) have reported an enhanced sensitivity of L-929 cells to TNSCT in the presence of actinomycin D. Similar results were demonstrated with PLCCT and BMCCT. Target cells demonstrated up to 250 times more sensitivity to CT in the presence of actinomycin D than if actinomycin D was not employed (data not shown).

Target Cell Susceptibility Profiles of Rabbit Macrophage Cytotoxins and Tumor Necrosis Serum Cytotoxin

Table IX compares PLCCT and BMCCT with TNSCT target cell susceptibility. Both macrophage derived cytotoxins are not toxic for mouse embryo fibroblasts or $B16C_3$ melanoma, two types of TNSCT-resistant cell, while L-929 cells, a TNSCT-sensitive cell, is also sensitive to macrophage CT. Chromium^{51} release studies demonstrated L-929 sensitivity to PLCCT and BMCCT, while $B16C_3$ cells were resistant. L929 cells released up to 18% of their label by 8 hours (taking into consideration spontaneous release of label by target cells) while $B16C_3$ cells released none of their label by 21 hours (data not shown).

TABLE IX

CELL SUSCEPTIBILITY PROFILES OF RABBIT CYTOTOXINS[a]

Target Cell	Cytotoxin Source	S_{50} Titer[c]
L-929	TNS[b]	>128
	PLC[b]	>128
	BMC[b]	>128
Mouse embryo fibroblasts	TNS	<2
	PLC	<2
	BMC	<2
$B16C_3$	TNS	<2
	PLC	<2
	BMC	<2

[a]Photometric assay without added actinomycin D was used to titer the same aliquot of each cytotoxin on the cells indicated.

[b]TNS, tumor necrosis serum; PLC, pulmonary lavage cells; BMC, bone marrow cells (TPA pretreated).

[c]Forty-eight hour titers.

Table X summarizes biological and physicochemical characteristics of rabbit macrophage cytotoxins and TNSCT.

Cocultivation of Mouse Peritoneal Exudate Cells or Rabbit Pulmonary Lavage Cells with Actinomycin D Pretreated L-929 Targets

Target cells can be rendered increasingly sensitive to TNSCT (a putative macrophage product) by actinomycin D (Ostrove and Gifford, 1979; Ruff and Gifford, 1981). In addition, Kunkel and Welsh (1981) have reported that pretreatment of L-929 cells with actinomycin D followed by washing rendered them increasingly sensitive to lysis by natural killer cells. Considering these observations, it was of interest to determine what effect actinomycin D pretreatment of L-929 cells followed by washing would have on macrophage mediated killing of L-929 targets. Table XI illustrates increased sensitivity of L-929 cells to mouse PEC cytotoxicity using a ^{51}Cr release assay. By 6 hours, nearly 40% of the ^{51}Cr was released by actinomycin D pretreated targets cocultured with LPS activated PEC, while by 12 hours, those targets not pretreated with actinomycin D released less than 10% of their label in the presence of effector cells. In this experiment, the effector:target cell ratio was between 1:1 and 10:1 (see Materials and Methods).

Demonstration of Cytotoxicity of Actinomycin D Pretreated L-929 Cells with Effector:Target Ratios <1

Actinomycin D pretreatment and washing of monolayers was followed by addition of small numbers of mouse PEC or rabbit PLC activated by the addition of LPS. Significant levels of killing could be detected by 18 hours using the photometric assay (see Figure 13). If actinomycin D was omitted, no killing was detected at 18 hours. It was possible to determine the number of effector cells required to kill 50% of the

TABLE X

SUMMARY: COMPARISON OF PHYSICOCHEMICAL AND BIOLOGICAL PROPERTIES OF RABBIT TUMOR NECROSIS SERUM CYTOTOXIN (TNSCT) WITH RABBIT MACROPHAGE CYTOTOXINS

Property	Cytotoxin (CT)			
	TNSCT	PLC[a]	BMC[b]	BLM[c]
Molecular weight, gel filtration	48K	48K	48K	48K
Elution from DEAE Sephadex	0.30-0.32M	0.28-0.30M	0.30-0.31M	0.32M
Glycoprotein	No[d,e]	No[f] ?	ND[g]	ND
Temperature stability: 56°C, 60 min.	All stable			
70°C (after 20 minutes)	All unstable			
Trypsin inhibitors (soybean and bovine pancreatic)	NI[h]	NI	NI	ND
Phenylmethyl sulfonylfluoride	NI[e]	NI	ND	ND
o-Phenanthrolene	All inhibited			
Chloroquine	All inhibited			
Arginine, 2 x 10^{-2}M	No Effect	No Effect	No Effect	ND
Cytotoxicity Assay with actinomycin D	All activities enhanced			
Target cell cytotoxicity: L-929	Susceptible to all			
$B16C_3$	Resistant to all			
Mouse embryo fibroblasts	Resistant to all			

[a]Pulmonary lavage cells

[b]TPA-pretreated bone marrow cells

[c]Blood monocytes

[d]Matthews et al., 1980

[e]Ruff and Gifford, 1981

[f]Tunicamycin did not decrease molecular weight of CT produced by PLC

[g]Not determined

[h]Not inhibited

TABLE XI

^{51}Cr RELEASE BY L-929 CELLS COCULTURED WITH MOUSE PERITONEAL EXUDATE CELLS

Time (hours)	Percent ^{51}Cr Released[a,b]	
	Actinomycin D Pretreatment	No Actinomycin D Pretreatment
3	1.3 ± 0	0
6	38.3 ± 1.8	1.9 ± 0
12[c]	46.8 ± 1.3	9.4 ± 0

[a]Percent specific ^{51}Cr released into supernatants = $\frac{E - S}{T - S}(100)$

where E = experimental release, S = spontaneous release and T = total releasable counts.

S = amount of ^{51}Cr released by target cells cultured alone.

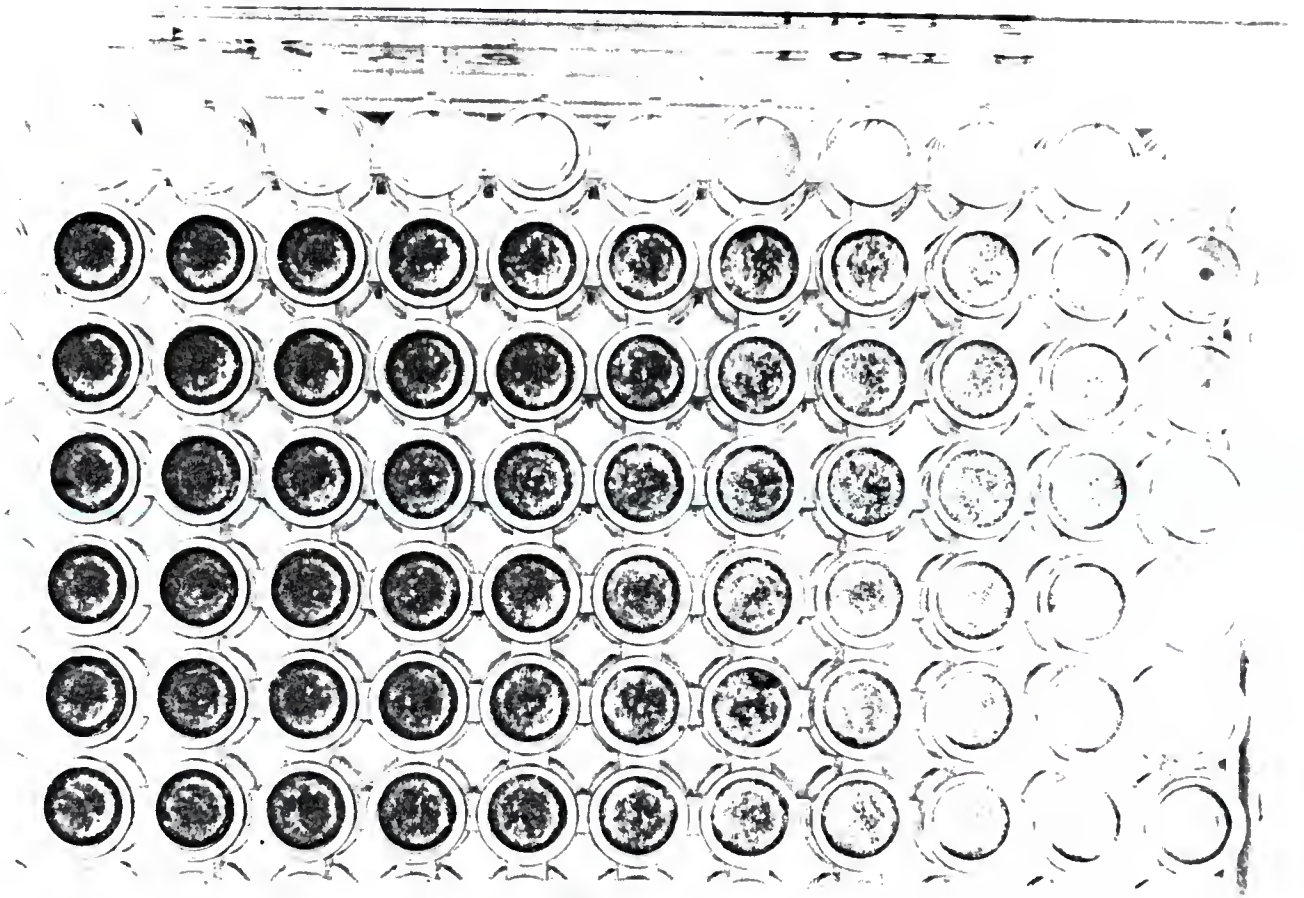
T = total releasable counts by lysis of 3×10^4 ^{51}Cr labelled L-929 cells with 1% dodecyl sodium sulfate.

[b]Samples run in triplicate for determination of experimental and spontaneous counts released. Samples run in quadruplicate for total releasable counts.

[c]Maximum spontaneous counts released = 22% of total releasable counts by 12 hours.

Figure 13. C_3H/HeN mouse peritoneal exude cells on actinomycin D pretreated L-929 cells: ninety-six well tray.

L-929 monolayers were pretreated with actinomycin D for 2 to 3 hours and then washed. Mouse peritoneal exudate cells were plated into the wells in 2-fold increments (numbers below the well columns indicate number of peritoneal exudate cells plated in 0.2 ml). LPS was added at 10 µg/ml. Eighteen hours later the plate was stained. Two sets of peritoneal exudate cells were run in triplicate.



0 10 20 40 80 160 320 640 1280 2560

C_3H PERITONEAL EXUDATE CELLS ON ACTINOMYCIN D

PRETREATED L 929 CELLS

targets. Figure 14 demonstrates this point. Approximately 175 rabbit PLC and 300 mouse PEC could kill 50% of the actinomycin D pretreated monolayers. If we take into account the fact that 95% of the rabbit PLC and 70% of the mouse (C_3H/HeN) PEC were macrophages, we can then determine the average number of targets killed by a single macrophage. Knowing that approximately 50,000 L-929 cells composed a monolayer, it is determined that a single rabbit macrophage killed an average of 25,000÷(175x0.95) or 150 targets and a single mouse macrophage killed an average of 25,000÷(300x0.70) or 119 targets. These are only average determinations, since, as will be shown, not every macrophage necessarily killed targets, nor were the number of targets killed by a single macrophage the same.

Controls for these experiments previously determined that the manual photocell employed for these studies could at no time detect the small number of effector cells plated. In addition, removal of adherent cell populations from mouse PEC virtually eliminated their ability to generate killing. We therefore concluded that the macrophage was the effector cell in the PEC population.

Demonstration of Single Effector Cell Cytotoxicity: Plaque Assay

Repeating the same experiment on a larger monolayer surface (24 well plates) resulted in a greater dispersal of the effector cells. Here, killing was detectable by the formation of plaques (see Figures 15 and 16). If the number of effector cells plated was compared with the number of plaques that resulted, a linear relationship between the two was obtained (Figure 17). This dictates that a single effector cell was capable of forming a single plaque, though not every effector cell

Figure 14. Effector cell cytotoxicity for actinomycin D pretreated L-929 targets: Determination of S_{50} endpoint.

L-929 monolayers were pretreated with actinomycin D (2µg/ml) for 2 to 3 hours. After washing monolayers, effector cells (a, mouse peritoneal exudate cells; b, rabbit pulmonary lavage cells) were added in 2-fold increments. Control target monolayers received no effector cells. LPS was added at 10 µg/ml. Target monolayers were stained after 18 hours.

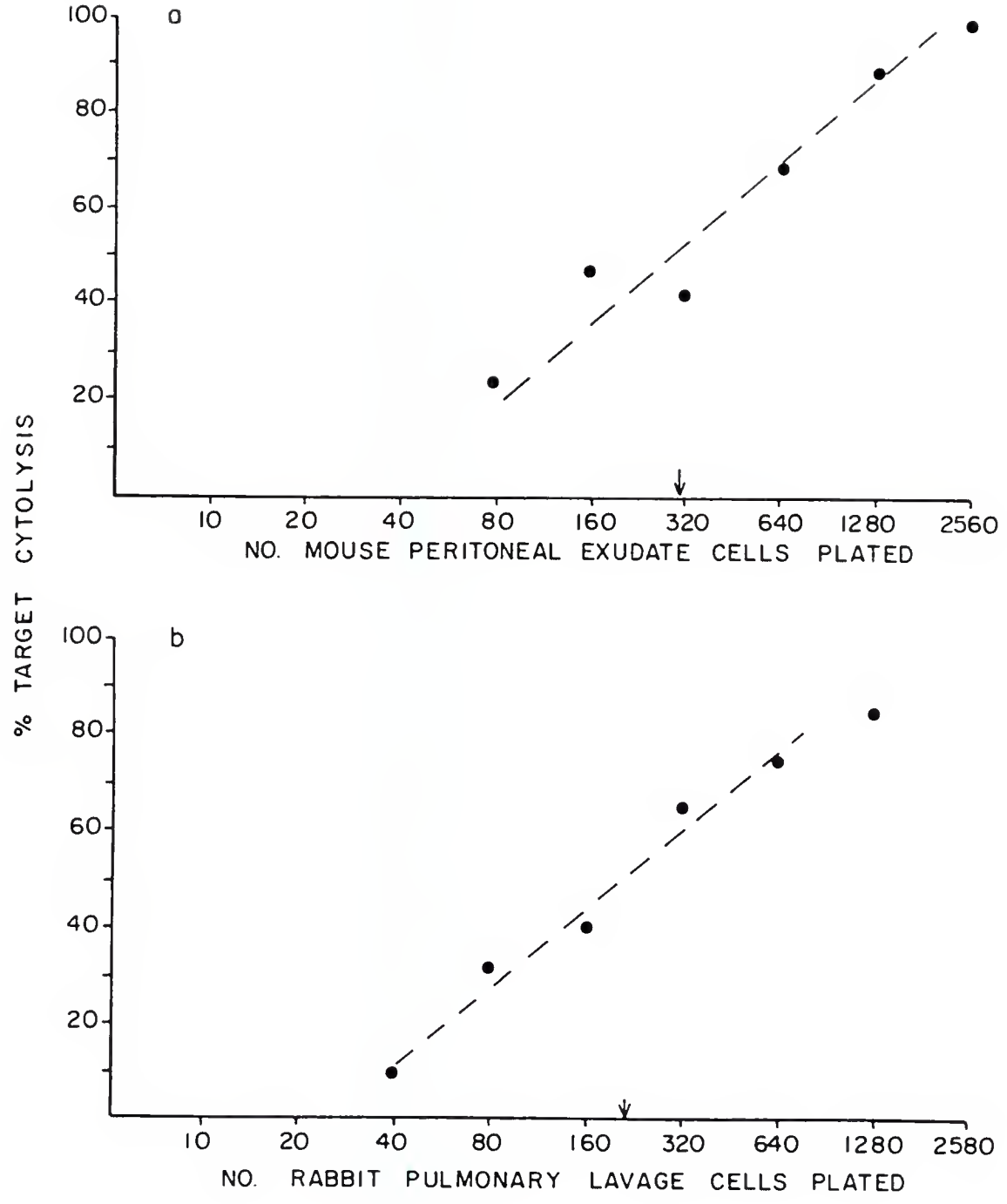
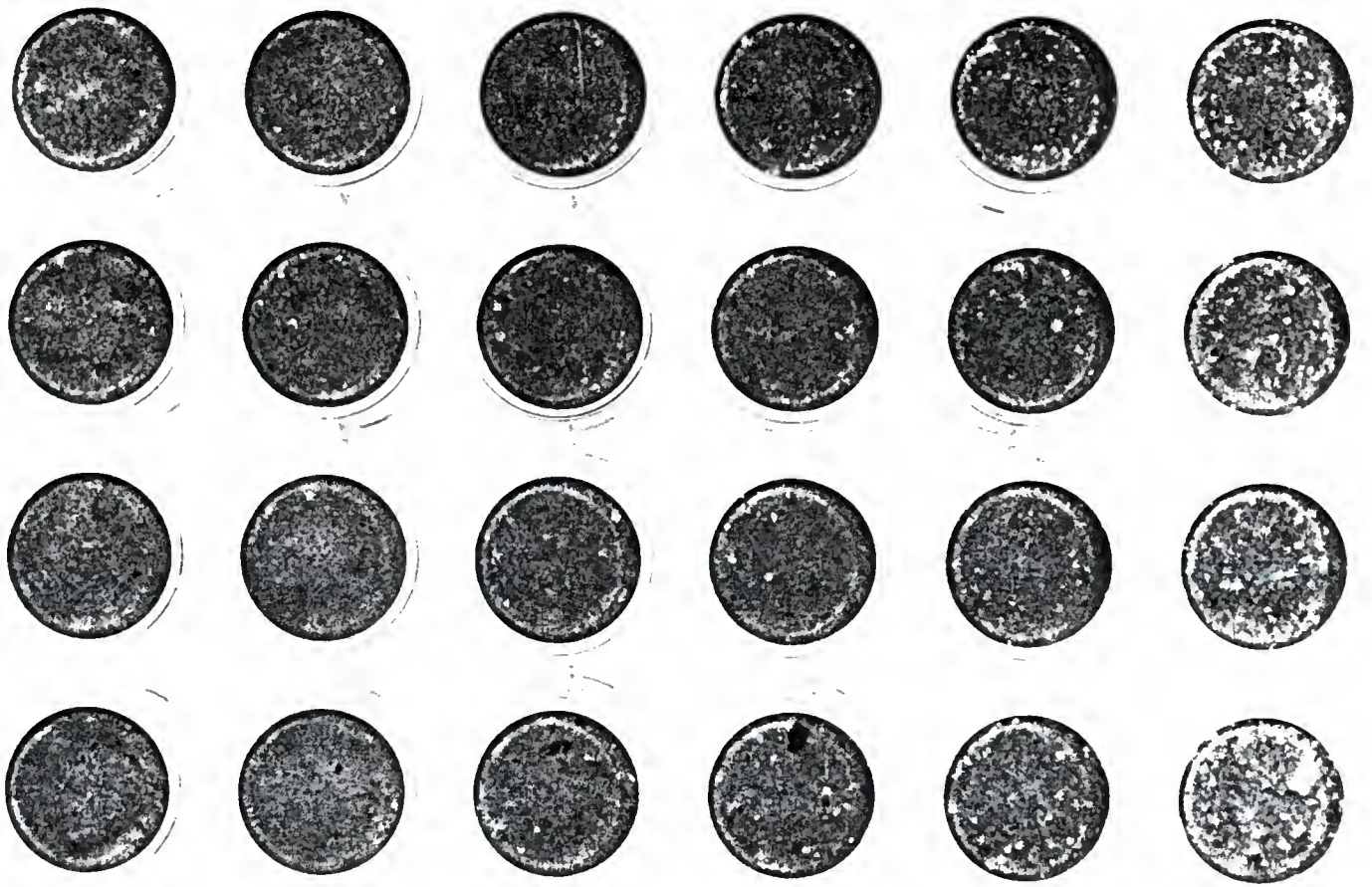

a
100
80
60
40
20
% TARGET CYTOLYSIS
10 20 40 80 160 320 640 1280 2560
NO. MOUSE PERITONEAL EXUDATE CELLS PLATED
b
100
80
60
40
20
10 20 40 80 160 320 640 1280 2580
NO. RABBIT PULMONARY LAVAGE CELLS PLATED

Figure 15. C_3H/HeN mouse peritoneal exudate cells on actinomycin D pretreated L-929 cells: Twenty-four well tray.

Procedure was the same as for peritoneal exudate cells on L-929 targets, 96 well tray (Figure 13). Numbers below the well columns depict number of peritoneal exudate cells plated in 0.2 ml. Each number of peritoneal exudate cells run in quadruplicate.

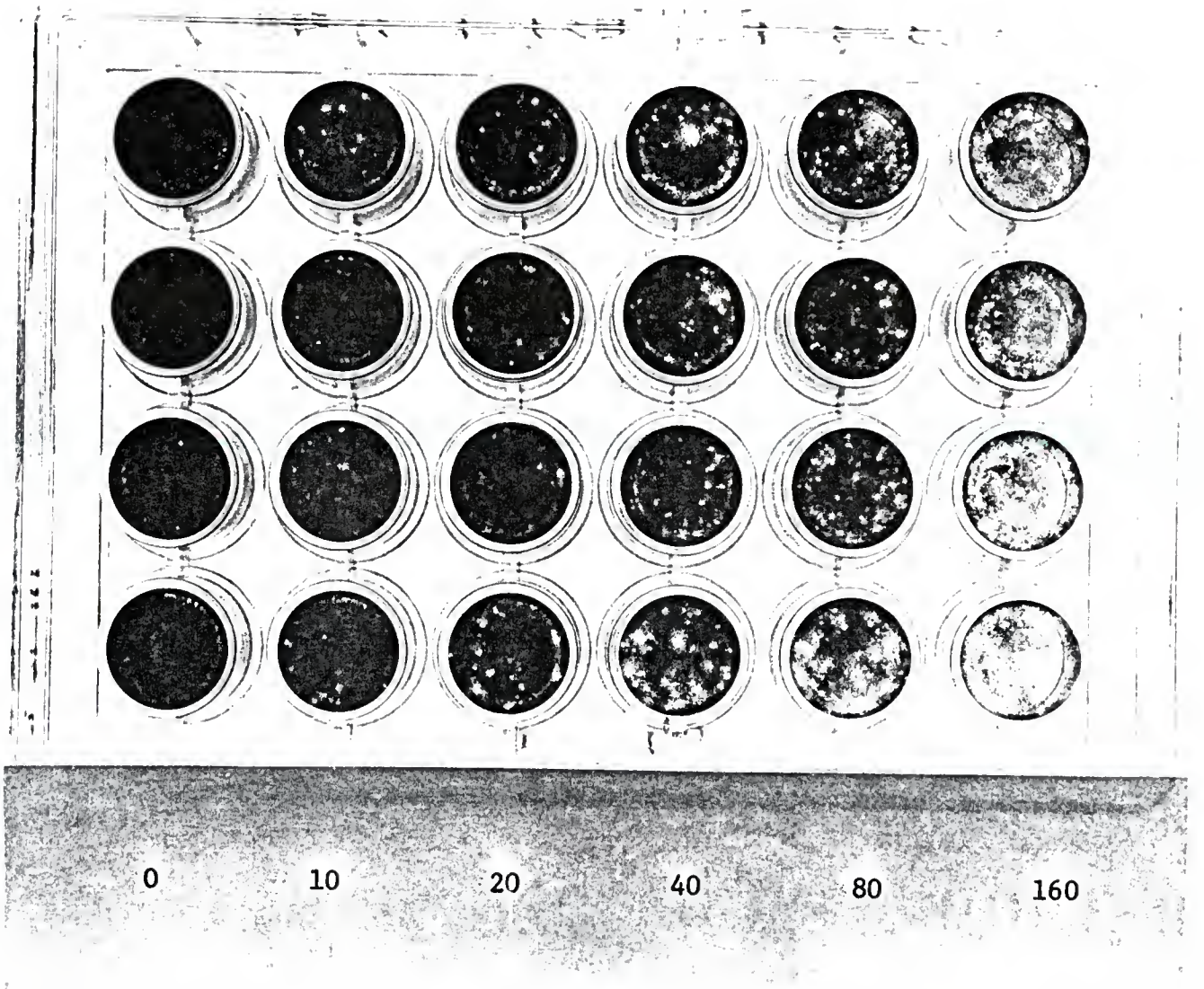


0 10 20 40 80 160

C_3H PERITONEAL EXUDATE CELLS ON ACTINOMYCIN D PRETREATED L 929 CELLS

Figure 16. Rabbit pulmonary lavage cells on actinomycin D pretreated L-929 cells: Twenty-four well tray.

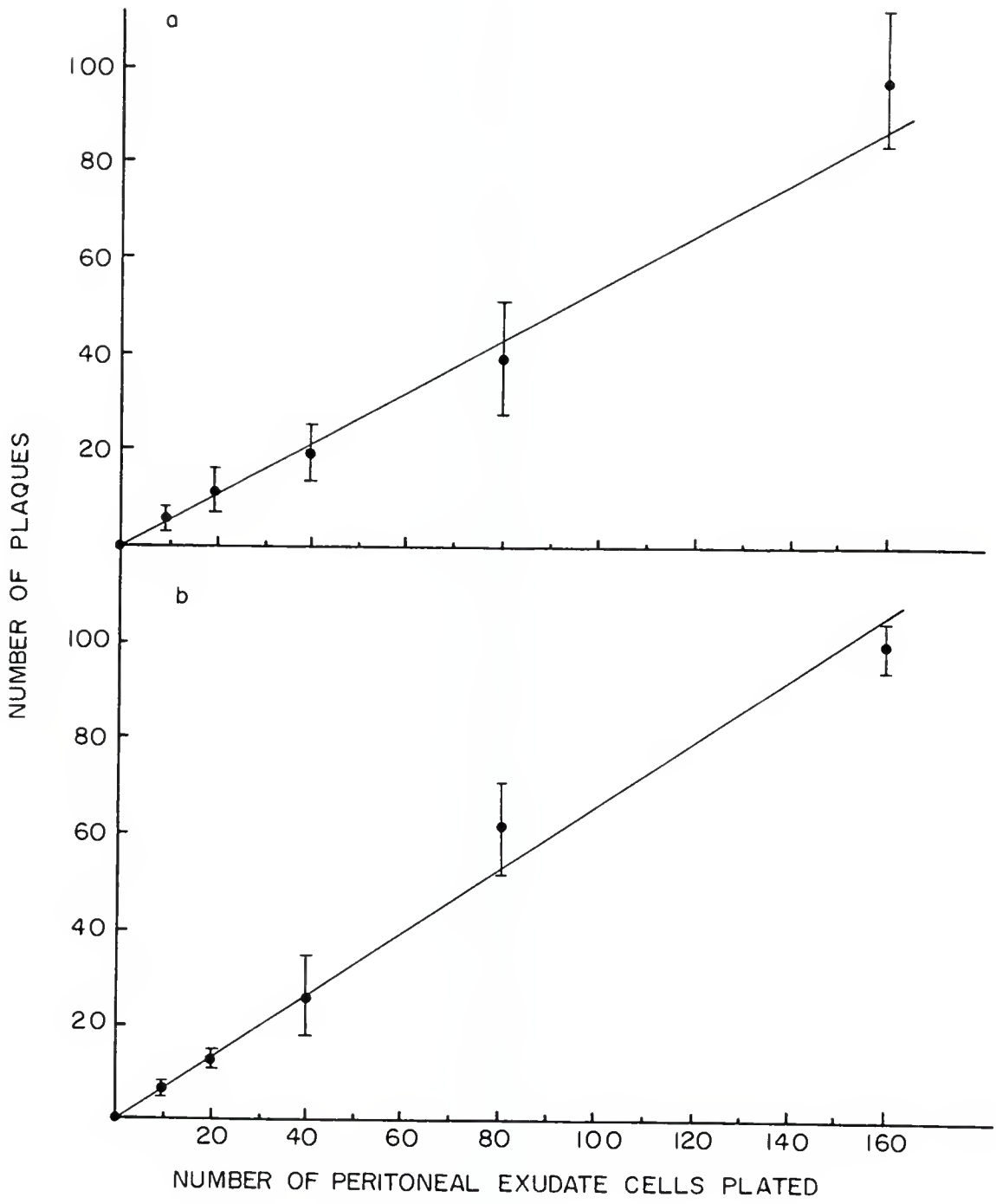
Procedure as for mouse peritoneal exudate cells (Figures 13 and 15).



RABBIT PULMONARY LAVAGE CELLS ON ACTINOMYCIN D PRETREATED L 929 CELLS

Figure 17. Linearity between number of plaques counted vs. number of mouse peritoneal exudate cells plated.

Each point represents the mean of 4 replicates ± 1 standard deviation. Panel a, represents peritoneal exudate cells from C57B1/6 mouse; panel b, represents peritoneal exudate cells from C_3H/HeN mouse. Both lines represent the best fit by linear regression and have correlation coefficients of >0.990.


a
b
100
80
60
40
20
20
40
60
80
100
120
140
160
NUMBER OF PLAQUES
NUMBER OF PERITONEAL EXUDATE CELLS PLATED

necessarily formed a plaque. Therefore, one plaque represented the killing events associated with a single macrophage.

For convenience, this assay was developed in mice and then utilized in the rabbit system. One of the most noticeable observations about the rabbit system concerns its variability (sensitivity of PLC to LPS when comparing different rabbits; see Figures 3 and 6). This plaque assay was performed using PLC from several rabbits. In only about 20% of the rabbits used did the number of plaques generated conform closely with the number of PLC plated. This probably demonstrated variation among rabbits concerning the number of macrophages in the overall PLC population that could be sufficiently activated to make a plaque. In addition, as in the mouse system, plaques varied in size, demonstrating variation in the ability of each macrophage to kill targets.

Other observations made in the rabbit plaque system included the inability of 2×10^{-2}M arginine in the medium to inhibit PLC plaque formation and the requirement for the addition of LPS to get plaques. L-929 cells for these latter studies were maintained in a culture system which tested negative (<0.025 ng/ml) for LPS (see Table III). Finally, if actinomycin D was added at the same time as PLC and LPS to actinomycin D pretreated and washed L-929 monolayers, no plaques were produced. However, if actinomycin D was added to the system 2 hours or more after the PLC and LPS were added to actinomycin D pretreated and washed monolayers, the same number of plaques were formed when compared to controls to which no actinomycin D was added either with or following PLC (data not shown).

Time Lapse Cinematography of Small Numbers of Rabbit PLC on Actinomycin D Pretreated L-929 Cells

Using the conditions described in Materials and Methods for cocultivation of effector cells on actinomycin D pretreated targets, time

lapse cinematography of small numbers of rabbit PLC killing L-929 cells demonstrated three main points. First, the PLC did not undergo much translational motion. They grazed locally, touching no more than 4 to 6 target cells on the average. Secondly, target cells closest to each PLC died first (about 6 to 8 hours after PLC and LPS were added to actinomycin D pretreated and washed targets). Finally, many target cells could be seen dying that were never touched by a PLC. All of this implies that single effector cells secreted sufficient CT to kill target cells and that a visible plaque represented for the most part, secretion of CT by individual PLC.

Cocultivation of Effector Cells with L-929 Targets not Pretreated with Actinomycin D

Hibbs (1976) has demonstrated that macrophage mediated target killing can be seen in 48 to 60 hours in a visual assay similar to our photometric assay minus the actinomycin D pretreatment. However, he employed high effector:target ratios (10 to 100:1).

Attempts to demonstrate target killing by low numbers of rabbit PLC or mouse PEC without pretreating the L-929 cells with actinomycin D have been unrewarding. At least 2000 to 5000 effector cells were necessary to produce toxicity for L-929 cells in the 96 well plate assay and in addition, the results were highly variable and difficult to quantitate. Furthermore, all attempts to generate plaques over a 48 to 60 hour period in 24-well plate assays (without actinomycin D pretreatment) have met with great technical problems. The few times that monolayers could be

generated that would survive under these conditions, no plaques were seen.

Susceptibility of TNSCT-Resistant L-929 Cells to Rabbit Macrophage Cytotoxins and Pulmonary Lavage Macrophages

L-929 cells selected for their resistance to TNSCT by being grown in the presence of the CT displayed significantly greater resistance to PLCCT, BMCCT and PLC cytotoxicity (Table XII). Most intriguing is the observation that L-929 cells resistant to the cytotoxins also resisted killing by macrophages. Similar results were also obtained with $B16C_3$ cells (data not shown). The implications of this further underscore the possible significance of the CT in macrophage mediated tumor killing.

TABLE XII

EFFECTS OF TNSCT,[a] PLCCT,[b] BMCCT[c] AND PLC[d] ON TNSCT-RESISTANT[e] AND CONVENTIONAL L-929 CELLS

Cytotoxin or Cell	Dilution or Effector Cell Number	Percent Survival L-929[f]	
		Resistant	Conventional
TNSCT	1:20	25 ± 8	7 ± 2
	1:200	75 ± 5	48 ± 10
PLCCT	1:20	72 ± 3	51 ± 7
	1:200	89 ± 4	76 ± 4
	1:2,000	100 ± 3	91 ± 13
BMCCT	1:20	66 ± 3	51
	1:200	99 ± 2	83 ± 12
PLC	1,000	97 ± 3	46 ± 8
	100	100 ± 10	83 ± 3

[a]Tumor necrosis serum cytotoxin.

[b]Pulmonary lavage cell cytotoxin.

[c]TPA-pretreated bone marrow cell cytotoxin.

[d]Pulmonary lavage cells.

[e]Grown in the presence of TNSCT, 1:500 dilution for 10 days.

[f]Forty-eight hour survival rate compared to control which received no cytotoxin or PLC; TNSCT-resistant and conventional L-929 cells each had a set of controls.

DISCUSSION

These studies have demonstrated three major points to be discussed:

1. Macrophages derived from different tissue sources representing different degrees of maturity all produced a CT *in vitro* with similar biological and physicochemical characteristics to one another, as well as to TNSCT.

2. CT production was dependent upon the presence of an activating agent (LPS) and was similarly affected by culture conditions that could alter classical macrophage cytotoxicity (effects of serum, actinomycin D, prostaglandin). This demonstrated that classical macrophage cytotoxicity (utilizing the effector cell) correlated with soluble CT production. CT production may reflect an index of overall macrophage cytotoxicity.

3. Actinomycin D pretreatment of target L-929 cells greatly enhanced their sensitivity to all macrophage CT tested as well as to the macrophage. Taking advantage of this, CT production by individual macrophages could be demonstrated by formation of plaques on target L-929 cells.

The aforementioned first point clearly demonstrates that activated macrophages can secrete soluble CT *in vitro*. This is something which many investigators have been unable to demonstrate (Hibbs, 1976). There could be several reasons for failing to detect soluble CT liberated by an effector cell. The most obvious reasons include using an assay of

insufficient sensitivity, harvesting and assaying culture super natants long after labile CT activity is gone and employing an animal species whose macrophages may not secrete CT to the extent of another. In the system used in these studies, rabbit macrophages could consistently produce a stable CT that was detectable in a type of assay previously shown to enhance the sensitivity of TNSCT (Ruff and Gifford, 1981) and lymphotoxin (Eifel et al., 1975). The variable nature of the system (outbred animals and using PLC, cells that are in contact with the environment to some extent) affected only the sensitivity of effector cells to activating agent and resulted in CT production of varying titers. Nevertheless, CT production occurred each time, though to a variable extent.

To the extent examined, it appeared that CT produced by rabbit macrophages from differing sources (representing different degrees of differentiation) were similar to one another as well as to TNSCT. Although the data presented did not prove identity of all these CT, they strongly suggest that this could be the case. If not identical in every respect, these macrophage CT could represent a family of related molecules demonstrating a degree of heterogeneity. There is precedent for this in the lymphotoxin system (Hiserodt et al., 1976; Hiserodt and Granger, 1977).

On the other hand, concerning the mouse system, there have been some reports of macrophages producing CT with properties distinct from TNSCT (MacFarlan and White, 1980). This indicates that the macrophage may be capable of producing groups of CT with broad differences. This may be necessary in light of the fact that tumors can display resistance to TNSCT, one particular CT (demonstrated by this dissertation, Tables IX and XII and Matthews, 1978). By generating more than one type of CT, each with

perhaps a different mechanism of action, macrophages may be able to cover the gamut of resistance displayed by different tumors to particular CT.

The second point of interest generated by these studies concerns the relevancy of secreted CT to macrophage mediated tumor killing in general. This too has been the subject of much debate. Some investigators (Keller, 1980) are of the opinion that some secreted soluble CT (e.g., H_2O_2) probably play no significant role in extracellular target killing since the levels generated in vitro are generally too low to be toxic for target cells. However, this does not rule out the possibility that these same CT which might be ineffective once released from the effector cell, could be the mediator of effector cell target killing while still associated with the effector cell (contact mediated cytolysis or requiring close apposition of effector to target).

Regardless of the specifics of the mechanism of CT action (solubilized and extracellular or cell associated), the data presented in these studies clearly demonstrate that conditions which are known to affect overall macrophage cytotoxicity for tumors, also affect the production of CT in culture supernatants. Once again, the variability of the macrophage system employed (comparing data from different rabbits) was manifested by differing degrees of CT production under a given set of culture conditions. However, the overall patterns of activation by LPS, enhancement by fetal bovine serum, and inhibition by bovine serum, PGE and actinomycin D were maintained when comparing data from similar experiments involving different rabbits.

Some specifics about some of these experiments warrant mentioning. Prostaglandin was added to rabbit PLC cultures 6 hours after addition of LPS. This was done since it had been determined in the mouse system

(Taffet and Russell, 1981) that macrophages require several hours in culture to become sensitive to the inhibitory effects of PGE. Whether this is the case for rabbit macrophages has not yet been determined. Nevertheless, considering the lability of PGE, it was probably wise to add it at some time after the activation signal and in a serum-free system. The results of this experiment clearly demonstrated an inhibition of CT production.

It was demonstrated that in vitro CT production by PLC (and TPA-pretreated BMC) was completed by 30 hours. Adding fresh medium with LPS at that time did not result in further production of significant CT levels, although viability of effectors was high at this time (>95%) and remained as such for at least 24 additional hours in culture. Whether this cessation of CT production was mediated by PGE or not was not determined, but regardless of the mechanism, this observation is important in demonstrating that macrophages may only have limited temporal cytotoxicity capabilities despite the continued presence of activating signal. This may represent another significant reason why tumors, in vivo, can escape destruction by activated macrophages.

The fact that actinomycin D could inhibit virtually all CT production if added at the same time as LPS, most likey indicates that no CT messenger RNA was present in PLC prior to activation (as well as no active CT). Another possibility may involve an inactive or strongly cell associated protein precursor which must be activated or released by a second protein translated from newly transcribed messenger RNA. In any event, messenger RNA synthesis is required for CT production during macrophage activation.

The third major point to be discussed involves the increased sensitivity of L-929 cells to PLC cytotoxicity, if the former were pretreated with actinomycin D, then washed prior to coculturing with effector cells. This increased target cell sensitivity induced by actinomycin D pretreatment has also been demonstrated for lymphotoxin (Eifel et al., 1975), natural killer cells (Kunkel and Welsh, 1981), and antibody and complement (Segerling et al., 1975) mediated cytotoxicity. Implicit in all this is the presence of some transcription-dependent general "repair mechanism(s)," which can aid target cells' survival in the presence of CT or cytotoxic effector cells.

Taking advantage of the great increase in sensitivity to effector cell mediated lysis brought about by this regimen, it was demonstrated that a macrophage could kill many target cells. This was demonstrated both indirectly (culturing low effector:target ratios in 96 well trays) as well as directly (plaque assay in 24 well trays).

The plaque assay in itself demonstrated by these studies presents many benefits to the investigator studying cell mediated cytotoxicity. Many of the previously described _in vitro_ techniques developed for the study of effector cell mediated tumor cytotoxicity demonstrate target cell killing by populations of effector cells. The most commonly used assays include release of radiolabelled compounds from prelabelled targets (Brunner et al., 1968; Meltzer et al., 1975), and measuring significant loss of adherent target cell monolayers (Takasugi and Klein, 1970; Weinberg et al., 1978). These techniques generally require relatively large numbers of effector cells while depending upon a high effector:target ratio (E:T) to detect killing of targets. As a result,

they do not allow one to study cytotoxic events brought about by individual effectors.

Some previously described assays do allow observations of individual effector cell cytotoxicity. The Jerne plaque assay (Jerne and Nordin, 1963) demonstrates antibody production to a specific antigen by a single B lymphocyte. Modifications of this assay have allowed the demonstration of specifically sensitized cytotoxic T lymphocytes. These are depicted as microscopic plaques on specific target cell monolayers (Bonavida et al., 1976) or conjugated effector-target cell pairs, many containing a killed target cell as illustrated by uptake of eosin or trypan blue dye (Grimm and Bonavida, 1979).

For the study of nonantibody producing cells, these latter assays have many shortcomings. In particular, among other problems, it is difficult to distinguish between the effector mediated cytolytic events (microscopic plaques) and the usual degree of spontaneous dying that cells can undergo (Bonavida et al., 1976). Aside from being cumbersome, the single target cell lysis technique depends upon direct contact between effector and target. This may not be suitable for proper interpretation of results where significant levels of cytotoxins may be secreted by effector cells, obviating the need for contact.

Concerning the plaque assay, the linear relationship between the number of effector cells plated onto target monolayers and number of plaques that resulted demonstrate that one effector cell (macrophage) was capable of generating a plaque, thereby detecting cytotoxicity associated with a single effector cell. This convenient assay allows for enumeration of effectors, can be completed in less than 24 hours and demonstrates killing that can be directly detected with the unaided eye.

This avoids the problem of distinguishing between a more subtle cytotoxic event mediated by effector cells and spontaneous loss of targets. The fact that individual effector cells were being detected can allow for the determination of any heterogeneity which can be present in the effector cell population, concerning number of effectors in a given population which can kill target cells and the degree with which each effector can kill. This was seen in this study. Effector cells (PLC) taken from different rabbits always demonstrated plaques of varying sizes. In addition, the number of effector cells in the overall population that were capable of generating plaques varied from about 30 to 100%. The number of target cells killed by a single macrophage could be estimated from the approximate sizes of the plaques and knowing how many target cells constituted the monolayer (or the calculated area occupied by a single target cell). This number (100 to 500) is within the same order of magnitude as that calculated previously for the experiments done in 96 well trays (see Results).

The implication of this is that a plaque, for the most part, must represent the secreted CT of a single effector cell, since it is unlikely that a macrophage could have moved so far from its original point of contact with the monolayer and in such a pattern. The time lapse cinematography was quite revealing along these lines. Macrophages only grazed locally, coming into contact with no more than 4 to 6 target cells. In addition, target cells which at no time were in contact with macrophages, were seen dying. From this it can be concluded that formation of a plaque visible to the unaided eye is represented by the secretion of CT by an individual effector cell.

This clearly demonstrates that under the proper culture conditions, secretion of CT can even be detected at the single effector cell level. This emphasizes the reality of the cell free CT in this system and its potential for killing tumor cells. The question which must be raised concerning this is the reality of such a situation in vivo. Although high levels of CT and TNF can be demonstrated in the serum of previously manipulated animals (see Introduction), it must be recalled that the conditions used to induce this state are incompatible with life. Therefore, it is more likely that far more modest levels of CT are usually generated during more "normal" circumstances. Keeping this in mind, along with the ability of a particular CT to kill a (sensitive) tumor, one can envision three conditions that would favor CT concentration in a given area to remain sufficiently intense to kill tumor cells. They are adequate macrophage density, sufficient activation of these macrophages, and the presence of naturally occuring agent(s) that would enhance a tumor's sensitivity to CT. Macrophages in varying numbers and states of activation within or near tumors have been described (Eccles and Alexander, 1974; Russell et al., 1976; 1977). The presence of naturally occurring factors which sensitize tumor cells to CT has yet to be described. The advantage this could confer on the tumor bearing host would be to enhance the "reach" of its macrophages and extend the limits of the macrophage microenvironment for killing tumor cells. This could be quite significant for survival of the host.

Another interesting point demonstrated by these studies concerns the resistance of TNSCT-resistant L-929 cells and $B16C_3$ cells to PLCCT, BMCCT, and PLC. The resistance of these cells to the soluble macrophage CT is consistent with the other data previously presented which

demonstrated strong similarity (possible identity) among these CT. More interesting is the remarkable resistance of the TNSCT-resistant targets to macrophages (Table XII). Implicit in this is that CT plays a significant role in overall macrophage mediated target killing and that other possible mechanisms are no more significant. It is possible that the effector PLC in these cultures did not secrete much soluble CT, but killed target cells primarily via a contact mediated mechanism that employed cell associated CT. This is suggested by the fact that virtually all the TNSCT-resistant targets survived in the presence of enough PLC (1000) that killed about half the conventional targets. Soluble PLCCT (as well as other CT tested) on the other hand, while killing an equivalent amount of conventional cells (about 50%) at appropriate dilutions, did not spare all the TNSCT-resistant cells, but killed 25 to 30% of them.

Repeated freeze-thawing of activated PLC did not reveal the presence of a significant active intracellular pool of CT. In addition, culturing freeze-thawed cells on L-929 cells in the presence of actinomycin D revealed no cytotoxicity. This presents a puzzle as to where this CT resides in the cell, particularly if it can mediate, at least in part, contact mediated target killing. It is possible that this CT in cell associated form must be rendered functional by some active effector or target cell process during macrophage activation or subsequent passage directly onto or into a target cell during effector:target contact. Hibbs (1974b) has described such a system where macrophages, while in direct contact with target cells, demonstrate some active injection process. More work is needed to clarify these points.

Mannel (1981) has drawn similar conclusions in the mouse system using a rabbit antiserum to TNSCT that could neutralize CT from PEC. In this mouse system, PEC could be primed in vivo to kill target cells in vitro without the presence of LPS. Nevertheless, these cytotoxic macrophages secreted no detectable CT into the medium, but their cytotoxicity for targets could be inhibited to a variable but never total extent (33 to 89%) by TNSCT antiserum. This also implied that the CT in this system was not totally responsible for macrophage mediated tumor cytotoxicity.

In conclusion, these studies have demonstrated macrophage mediated tumor cytotoxicity from the point of view of a secreted cytotoxic molecule. Whereas this may be an oversimplification of a very involved process due to limited knowledge concerning the intracellular state of this moiety, it does represent a beginning of sorts in assigning at least part of a cellular function to a molecular species. By studying how this CT can distinguish between the tumor and nontransformed state and by learning more of its association with the macrophage, we may better understand why neoplasms can still escape such intricate mechanisms and contribute much to the frustration accompanying efforts to better comprehend and treat cancer.

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BIOGRAPHICAL SKETCH

Harvey Fisch was born 17 September, 1946, 3:15 A.M. in Brooklyn, New York. After graduating from Erasmus Hall High School, Harvey left Brooklyn to attend Cornell University in frigid Ithaca, New York. After receiving his Bachelor of Science degree in microbiology in June of 1968, he remained at Cornell another four years while attending the New York State College of Veterinary Medicine. After receiving the Doctor of Veterinary Medicine degree in 1972, he entered private practice for two years in New Jersey and Florida. From 1974 to 1976, he served in the U.S. Army as a general veterinary officer and was stationed at the U.S. Army Environmental Hygiene Agency, working in laboratory animal medicine and toxicology. After pushing papers for two years, he left active duty (to be honorably discharged later) and entered graduate school at the University of Florida, department of Immunology and Medical Microbiology, in 1976.

For three years, he was a graduate and teaching assistant. He was then awarded a two year postdoctoral fellowship by the American Cancer Society. He has just passed his board certification examination in veterinary microbiology, conferring upon him Diplomate status in The American College of Veterinary Microbiologists. He plans to continue his research in his current project for a while following graduation before pursuing other endeavors.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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